THE EFFECTS OF CHRONIC RESTRAINT STRESS ON INNATE AND ADAPTIVE IMMUNE RESPONSES TO ACUTE THEILER'S MURINE ENCEPHALOMYELITIS VIRUS INFECTION – AN ANIMAL MODEL OF HUMAN MULTIPLE SCLEROSIS

A Dissertation

by

ANDREW STEELMAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2008

Major Subject: Biomedical Sciences

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ABSTRACT

The Effects of Chronic Restraint Stress on Innate and Adaptive Immune Responses to Acute Theiler's Murine Encephalomyelitis Virus Infection – An Animal Model of Human Multiple Sclerosis. (May 2008)

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Multiple sclerosis (MS) is an immune-mediated prevalent chronic demyelinating and neurodegenerative disease of the central nervous system that begins with an abrupt onset during early adulthood. MS is idiopathic, but many factors are thought to influence the pathogenesis of the disease, which include genetic, gender and environmental factors. To date, there is much evidence that suggest that both the onset and progression of MS is facilitated by both viral infections and stress. Theiler's murine encephalomyelitis virus (TMEV) is a picornavirus that upon inoculation into susceptible strains of mice (i.e. SJL and CBA) causes a persistent infection which, in turn, results in an early acute encephalomyelitis followed by a late chronic immune-mediated demyelinating and neurodegenerative disease that pathologically resembles MS. In contrast, resistant mice (i.e C57BL/6 and BALB/c) are able to clear the virus from the CNS, and consequently do not develop chronic demyelination. Previous studies indicated that stress during early infection of susceptible mice can increase CNS viral titers and alter dissemination of TMEV, decrease early cytokine and chemokine

expression in the spleen and CNS, and result in an exacerbated late demyelinating disease. The studies herein, focused on the hypothesis that chronic stress during early infection with TMEV infection would lead to drastic immunosuppression of both innate and adaptive arms of immunity, and that this immunosuppression may overcome the genetically controlled resistance of C57BL/6 mice to Theiler's virus-induced demyelination. In these series of studies, we were able to show that stress, regardless of mouse strain susceptibility, decreases NK cell activity, and increased viral titers at day 1 p.i. Furthermore, after seven days of stress, susceptible mice demonstrated decreased virus specific T-cell effector function in both the CNS and spleens as indicated by a globalized reduction in type 1 and type 2 cytokines, as well as transcription factors. Importantly, these decreased responses were, in part, attributable to the actions of glucocorticoids. However, stress during early infection of C57BL/6 mice did not alter resistance to demyelination. These results begin to shed light on how stress, infection, and genetics can influence the onset of human MS.

DEDICATION

This document is dedicated to the Brazos Valley MS support group, my loving and understanding wife Samantha, my family and friends who have supported me and encouraged me to pursue happiness in life.

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NOMENCLATURE

ACTH	Adrenocorticotropin-Releasing Hormone
AP-1	Activator Protein-1
BBB	Blood Brain Barrier
ChIP	Chromatin Immunoprecipitation
CNS	Central Nervous System
CRP	C-reactive Protein
CRH	Corticotropin-Releasing Hormone
CSF	Cerebrospinal Fluid
DC	Dendritic Cell
EAE	Experimental Autoimmune Encephalomyelitis
EBV	Epstein-Barr Virus
ELISA	Enzyme Linked Immunosorbent Assay
ELISPOT	Enzyme Linked Immunosorbent Spot
GC	Glucocorticoid
GCR	Glucocorticoid Receptor
HLA	Human Leukocyte Antigen
HPA	Hypothalamic-Pituitary-Adrenal
IFN	Interferon
IL	Interleukin
MBP	Myelin Basic Protein

MHC	Major Histocompatibility Complex
MOG	Myelin Oligodendrocyte Glycoprotein
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
NK	Natural Killer
PLP	Proteolipid Protein
PPMS	Primary Progressive Multiple Sclerosis
RRMS	Relapsing-Remitting Multiple Sclerosis
RS	Restraint Stress
SAA	Serum Amyloid A
SAP	Serum Amyloid P
SEM	Standard Error of the Mean
SFC	Spot Forming Cell
SNS	Sympathetic Nervous System
SPMS	Secondary Progressive Multiple Sclerosis
STAT	Signal Transducer and Activator of Transcription
T-bet	T-box Expressed in T-cells
Th	T-Helper
TMEV	Theiler's Murine Encephalomyelitis Virus
TMEV-IDD	Theiler's Murine Encephalomyelitis Virus-Induced Demyelinating Disease
VP	Viral Protein

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I INTRODUCTION

1. Multiple sclerosis

1.1 History and prevalence of multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory, neurodegenerative and almost always debilitating disease that is confined to the central nervous system (CNS) and thought to be mediated by autoimmune processes. Charcot is credited with the initial description of the disease in 1868 although others, in particular Carswell and Cruveiller, twenty years ealier described a similar disorder (Morales et al., 2006). While the disease was only first recognized as a specific entity approximately 170 years ago, it is most likely not a new disease, as historical records may provide evidence of its existence among the Vikings during the 14th century (Morales et al., 2006). Despite the fact that MS has been around for centuries, there is a general consensus that its incidence is, in fact, increasing (Noseworthy et al., 2000; Noonan et al., 2002; Hauser and Oksenberg, 2006).

Indeed, a recent worldwide assessment of the most common diseases of the CNS, which included MS, as well as others, indicated that MS had an incidence of 4.1 per 100,000 and a prevalence of 0.9 per 1000 (nearly 0.1% of the world population) (Hirtz et al., 2007). When considering these statistics, it is important to keep in mind that both the incidence and prevalence of MS is influenced greatly by latitude. This observation has recently been confirmed by an analysis of the incidence and prevalence of MS in

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Europe, where, for example, the authors demonstrate that the prevalence for Northern Scotland are 168 and 186 per 100,000 respectively, but only 50 and approximately 45 (32-58) for France and Spain (Pugliatti et al., 2006). In the United States (US) there also exists a gradient of MS prevalence that is dependent on latitude where the prevalence of MS is generally higher in northern states compared to southern states (Kurtzke, 1980). Nevertheless, the overall prevalence of MS worldwide is estimated at over 2.5 million (Compston and Coles, 2002) and the prevalence of MS in the US is estimated between 250,000 to 350,000 (Anderson et al., 1992; Hirtz et al., 2007). Even though the incidence and prevalence of MS is lower than other neurological diseases (Hirtz et al., 2007), the age of onset and debilitating nature of the disease ensure a high cost to the patient as well as society. For instance, MS has an estimated medical cost of over $2 billion per year in the UK (Homes et al., 1995) and $6.8 billion per year in the US (Whetten-Goldstein et al., 1998).

The onset of multiple sclerosis usually occurs between the ages of 15 and 40 (Kantarci and Weinshenker, 2005) although in rare cases individuals have certainly been diagnosed with MS before and after this timeframe. MS, like other autoimmune diseases including lupus, and rheumatoid arthritis, has a predilection for females rather than males which is illustrated by the fact that the odds ratio of being diagnosed with MS is 2:1 female to male (Noseworthy et al., 2000; Hirtz et al., 2007). However, males, for reasons unknown, present with a more exacerbated disease than females (Kantarci and Weinshenker, 2005).

1.2 Natural history of multiple sclerosis

For each case of MS, the natural history is extremely heterogeneous in nature (Noseworthy et al., 2000; Pugliatti et al., 2006). The initial symptoms at onset generally include, but are not limited to, optic neuritis, L'hermitte symptom, involuntary limb movement, ataxia, fatigue, incontinence, and vertigo (Noseworthy et al., 2000). As the disease progresses, patients may experience cognitive difficulty, sexual dysfunction, paralysis, gait ataxia, incontinence, pain, depression and fatigue (Noseworthy et al., 2000). Typically, the disease will take on a relapsing remitting (RRMS) course where the patient will experience a neurological symptom lasting 24 hours or more in duration, followed by nearly complete remission (Wingerchuk et al., 2001). This type of MS occurs in approximately 85% of patients (Kantarci and Weinshenker, 2005). During RRMS, relapses are associated with the appearance of hyperintensive T2-weighted lesions of the white matter in the CNS indicating edema and tissue damage, as well as hypointensive T1-weighted lesions indicating atrophy, and hyperintensive gadolinium (Gd) enhancing T1 weighted lesions indicating disruption of the blood-brain barrier (BBB) (Neema et al., 2007). These inflammatory events that occur during relapse are mirrored by the pathological findings within the lesion which include microgliosis (hypercellularity), activated macrophages, both lipid and myelin-laden macrophages, the presence of $CD4^+$ and $CD8^+$ T-cells, plasma cells, antibody and complement (Frohman et al., 2006). A heterogeneity between lesions of different MS patients has been described, and potentially characterizes patients into four groups based on the occurrence or absence of macrophage, T-cell, antibody, complement and apoptosis

within the lesion (Luccinetti et al., 1999; 2000). This heterogeneity between patients may suggest four distinct types of disease, but these findings await conformation as several groups have now demonstrated that more than one category of lesion can appear in one patient at the same time, which indicate that the results observed by Luccinetti may be attributable to the evolution of the lesion over time (Barnett and Prineas, 2004; Breij et al., 2008).

After a time, most patients will continue to develop secondary progressive multiple sclerosis (SPMS) which is characterized as a slow continuing progression of symptoms with few or no relapses and is generally unresponsive to treatment (Kantarci and Weinshenker, 2005). During this phase of the disease, it is believed that the symptoms progress due almost exclusively to neurodegenerative processes, which is apparent by the decreases in N-Acetylaspartate levels, a neuronal cell marker, by ^{1}H-MRS and by apparent "black holes" by T1-weighted MRI which correspond to increases in cerebral atrophy (Neema et al., 2007). Unfortunately, there is currently no apparent reason for the switch from RRMS to SPSM, although some indicators such as age, gender, and the appearance of more than one lesion at onset, can predict a more rapidly advance to SPMS (Kantarci and Weinshenker, 2005).

Interestingly, not all patients will progress in the above-mentioned natural history. Instead, about 10-15% will develop a primary progressive form of the disease (PPMS) in which the relapsing remitting phase is seemingly skipped (Miller and Leary, 2007). PPMS is thought to differ from RRMS in that patients generally have a later onset (40 years of age), coinciding with the time in which RRMS patients normally

progress to SPMS, and experience more symptoms consistent with motor impairment of the spinal cord rather than sensory impairment (Miller and Leary, 2007). Peculiarly, unlike RRMS, there is no gender predilection in PPMS (Miller and Leary, 2007).

Benign MS is characterized by a failure to exhibit clinical MS symptoms for decades, and in some cases the patients will never experience a secondary symptom (Ramsaransing and Keyser, 2006). The incidence of this disease varies widely depending on the geographic location studied (Ramsaransing and Keyser, 2006). Interestingly, these patients have shown to possess the same T2-lesion volume, but at the same time demonstrate almost no decreases in markers of neuronal loss (Ramsaransing and Keyser, 2006). The progression of multiple sclerosis is currently measured by the expanded disability status score (EDSS) designed by John Kurtzke (Kurtzke, 1983), and by MRI – the latter being more indicative of cognitive dysfunction.

1.3 Diagnosis of multiple sclerosis

For the clinician the diagnosis of MS is difficult for several reasons. First, the symptoms at onset are similar to other idiopathic disorders of the CNS. A perfect example is the presentation of optic neuritis (ON). While approximately 38% of patients with an initial presentation of ON will develop MS within ten years, some will develop neuromyelitis optica, but most will keep the diagnosis of ON, which is itself considered a separate idiopathic disease (Kantarci and Winshenker, 2005). Secondly, there are currently no disease markers available to test exclusively for MS. For instance, even though it is known that the occurrence of oligoclonal bands in the cerebral spinal fluid

(CSF) are indicative of MS and are, incidentally, used in the diagnosis of MS, these banding patterns are also present in virally-induced meningitis and as such cannot be relied on exclusively in the ascertainment of MS. Therefore, the diagnosis of clinically definite MS must adhere to the following criteria set forth by Poser (1983) and modified by McDonald (2001). The symptoms must show dissemination of lesions typical of MS between "time and space", and all other potential diseases must be excluded (Poser et al., 1983; McDonald et al., 2001). For example, a patient must demonstrate a least two relapses lasting no lessthan 24 hours in duration (time), that occur at least 30 days apart, each of which demonstrate different affected areas (space). If two separate relapses occur that cannot be designated to a different anatomical location by the presentation of clinical symptoms, visual evoked potentials (VEP) can be used to determine lesion within the visual pathway or MRI findings can substitute (McDonald et al., 2001). In fact, MRI analysis alone can provide evidence of clinically definite MS given that three out of four of the following are ascertained over time: one hyperintensive Gd-enhancing T1 or nine hyperintensive T2 lesions, at least one infratentorial lesions, at least one juxtacortical lesion, or at least three periventricular lesions (McDonald et al., 2001). Additionally, one spinal cord lesion that is typical of MS as seen on MRI can substitute for one brain lesion (McDonald et al., 2001). Oligoclonal banding in CSF can also provide evidence typical of MS, even though it has no bearing on the dissemination of lesions (McDonald et al., 2001). The diagnosis is reached when the above-mentioned criteria are met. Therefore, if a patient at presentation has had two or more attacks with clinical evidence of two or more lesions, no additional evidence is needed for a

diagnosis. However, if a patient at presentation has had one attack with MRI demonstrating two or more lesions, the patient still needs to demonstrate dissemination in time, which can be obtained by future MRI analysis or by a second clinical attack (McDonald et al., 2001). Alternatively, if the patient has had two or more attacks, but only one clinically apparent lesion, then the patient needs to still show dissemination in space, by MRI or two or more MRI lesions with a CSF that is positive for oligoclonal bands or by future clinical symptoms indicative of a different affected area (McDonald et al., 2001).

1.4 Biological markers of multiple sclerosis

The difficulty in achieving a diagnosis of clinically definite MS can be frustrating both to the patient as they face the uncertainty of the future of their disorder and to the clinician as they are forced to withhold potentially beneficial treatment until a diagnosis can be reached. Because the requirement of dissemination between time and space must be met before a diagnosis, and given that most patients only experience 1-2 relapses per year during RRMS, this process could take a substantial amount of time. Additional markers of MS are needed to be discovered that will expediate the process of obtaining a valid diagnosis as well as a prognosis.

On this note, the presence of serum IgM antibodies directed against either myelin oligodendrocyte glycoprotein (MOG), a component of myelin that has an Ig domain exposed to exogenous space, or myelin basic protein (MBP), a major constituent of myelin, have been reported to be of prognostic value and, in fact, correlated with Gd-

enhancing MRI findings (Berger et al., 2003). These results are still preliminary as later studies conducted by the same group and others have both supported and negated these initial observations (Reindl et al., 2006).

Other markers that may hold some promise in aiding diagnosis are the acute phase proteins: C-reactive protein (CRP) and serum amyloid A (SAA). CRP is the major human acute phase protein that can increase up to 10,000 fold within 24 hours following an inflammatory event and has a half-life of 19 hours (Pepys and Hirschfield, 2003). SAA, also found in humans, is the only molecule with the capability to fluctuate between such dynamic ranges as CRP (Pepys and Hirchfield, 2003). Both CRP and SAA have been reported to be increased in the serum of RRMS patients during relapse (Wei and Lightman, 1997; Ristori et al., 1998; Boylan et al., 2001), a finding that has recently been confirmed for serum CRP by Soilu-Hannieen et al., (2005). Also, it has been shown that over time CRP levels significantly correlate with change in brain volume (Giovannoni et al., 2001a) as well as correlate with fatigue in MS patients (Giovannoni et al., 2001b). However, it is important to mention here that acute phase proteins, as well as cytokines, reactive oxygen species (such as nitric oxide) and cell activation markers would never be specific as they are increased by nearly all inflammatory events – including atherosclerosis (Pepys and Hirschfield, 2003; Du Clos, 2003).

Another marker with potential in both diagnosis and prognosis is the NOGO-A isoform. NOGO-A is a recently discovered neuronal growth inhibitor that is present in abundance in myelin and oligodendrocytes (Chen et al., 2000; Kuhlmann et al., 2007).

Interestingly, Jurewicz et al. have demonstrated that the N-terminal region of the soluble form of the NOGO-A protein is detectable in the CSF of as many as 96% (110 of 114) of patients with MS (both RRMS and SPMS), 100% of patients with clinically isolated syndromes (11 of 11), and no patients with other neurological diseases (including meningo-encephalomyelitis, Creutzfeldt-Jakob disease, epilepsy, vascular event, cranial nerve palsies, and chronic inflammatory demyelinating polyneuropathy) or autoimmune diseases (systemic lupus erythematosus, and neuromyelitis optica) (Jurewicz et al., 2007). Moreover, this protein was detectable in the early and later stages of disease (Jurewicz et al., 2007). However, this finding awaits conformation.

1.5 Autoimmunity in multiple sclerosis

The major problem in the ascertainment of biological markers specific to MS is the fact that it is an idiopathic disease. Even though much is known about the immunology of MS (reviewed by Sospedra and Martin, 2005), it remains to be proven that MS is actually an autoimmune disease, since it is unethical to fulfill Witebsky's postulates. Witebsky's postulates for autoimmunity were proposed in 1957 following his observations on the role of auto-reactive antibodies in chronic thyroiditis, and are modeled after Koch's postulates (Witebsky et al., 1957). According to Witebsky, for autoimmunity to exist; an "active" autoantibody must be isolated from the circulation; the corresponding antigen must then be identified, and produce autoantibodies following injection into experimental animals; and must result in disease which resembles the human disorder (1957). Rose and Bona have since revised Witebsky's postulates to

include direct, indirect and circumstantial evidence of autoimmunity based on the current knowledge of molecular immunology (Rose and Bona, 1993). Here, for 100% confirmation (direct proof) of autoimmune-induced disease, the transfer of autoantibody or autoreactive T-cells into a recipient must produce disease – hence the above mentioned ethical problems (Rose and Bona, 1993). However, indirect proof of autoimmunity can be provided by: the pathogenic transfer of reactive T-cells to naive or severely combined immunodeficient (SCID) mice, the induction of disease in animals by autoantigen, the identification of inflammatory cells at the site of lesions, through genetic models, and by the presence of autoantibodies in serum (Rose and Bona, 1993). Finally, circumstantial evidence of autoimmunity is demonstrated by familial aggregation, lymphocyte infiltration of the target organ, statistical associations with MHC alleles, and responsiveness of patients to immunosuppressive treatments (Rose and Bona, 1993).

Even though there is no way to prove that MS is an autoimmune-mediated disease, there is substantial indirect as well as circumstantial evidence suggesting that the myelin damage within the MS lesion is brought about by autoinflammatory events. For example, some of the evidence for an autoimmune pathogenesis of MS includes the presence of autoreactive $CD4^+$ and $CD8^+$ T-cells in the CSF and plaques of MS patients, the presence of antibody in plaques and oligoclonal bands in CSF of MS patients, the association of inflammatory events around lesions, and lesions occurring in perivascular locations as well as periventricularly, indicating trafficking of cells into the CNS (Pugliatti et al., 2006). Lipid and myelin-laden macrophages at the site of the lesion as

well as the responsiveness of RRMS patients to immunosuppressive therapy (i.e. cyclophosphamide and glucocorticoids) during relapse also indicates autoimmunity (Frohman et al., 2006). Additionally, treatment with MBP in an attempt to induce tolerance in RRMS patients resulted in increased incidence of relapse (Bielekova et al., 2000). Another indication of autoimmune pathogenesis can be illustrated by the recent clinical trials with natalizumab, a humanized monoclonal antibody that recognizes the $\alpha_4\beta_1$ integrin on T-cells and prevents their entry into the CNS by impeding diapedesis across the BBB. This study found that in RRMS patients receiving treatment over the course of two years, 68% had reduced relapse rate and the hyperintensive T2-weighted lesion load was reduced by 83% (Polman et al., 2006).

Knowing about the pathogenesis of a disease may result in some effective treatments, but in order to develop an effective cure one must know the causation. As stated previously, the cause of MS is not yet known. However, multiple factors are thought to play a role in onset and exacerbation of the disease. These include gender, genetic and environmental factors.

1.6 Genetic influence on multiple sclerosis

The familial association with MS onset was first postulated by Eichhorst in 1896 (Dyment et al., 2004). Today there is little question pertaining to a genetic involvement with MS. For instance the λs, a measure used to estimate the degree of inheritability of a particular disease based on familial aggregation and population prevalence, is between 20 and 40 (Hauser and Oksenburg, 2006). Additionally, twin studies from both

Canadian (Ebers et al., 1986; Sadovnick et al., 1993; Willer et al., 2003) and Danish (Hansen et al., 2005a; Hansen et al., 2005b) groups indicate that the concordant risk among female monozygotic twins is between 25-30%, but only 3-5% among dizygotic twins. However, it is interesting to note that gender may still have an influential role even among monozygotic twins as Willer et al., reported the risk among females to be 34% ± 5.7% whereas in males the risk was 11.4% ± 7.4% (Willer et al., 2003). While the risk of occurrence is high among twins it quickly begins to approach that of the general population in half-siblings (Sadovnick et al., 1996), adoptees (Ebers et al., 1995), and spouses (Ebers et al., 2000), providing further evidence in support of a genetic association with MS.

While studies which focus on the familial aggregation of MS associate onset to an inheritable trait, linkage mapping studies have demonstrated that the genetic association with MS is extremely multigenic. This is best illustrated by Table 1-1 which shows statistically significant regions of the genome that have been associated with MS (Dyment et al., 2004). Indeed, all human chromosomes, with the exception of chromosome 21, display at least one locus that is associated with the disease (Dyment et al., 2004). Most recently, genome wide scans for single nucleotide polymorphisms (SNPs) of 931 family trios in which both parents and one child had MS versus. 2431 controls demonstrated multiple SNPs located within the IL-2Rα ($p = 2.96 \times 10^{-8}$), IL-7Rα, ($p = 2.94 \times 10^{-7}$) and HLA-DRA locus ($p = 8.94 \times 10^{-81}$) (Hafler et al., 2007). As these genes are strongly associated with $CD4^+$ T-cell responses, these findings provide circumstantial evidence for autoimmunity in MS.

Among the associated alleles for multiple sclerosis are several HLA class I alleles. In fact, the first genetic association with MS was determined to be the HLA-A3 allele (Jersild et al., 1972; Naito et al., 1972). These initial findings have been substantiated by Fogdell-Hahn et al. (2000), who demonstrated that 41% of MS patients (n = 200) were positive for HLA-A*0301 compared to 25% of controls (n = 210). Additionally, this group also noted a statistically positive association with the HLA-B*0702 allele, which was subsequently found to be in linkage disequilibrium with HLA-DRB1*1501 (see below) (Fogdell-Hahn et al., 2000). The most interesting finding by this group was their ability to demonstrate a negative association with the HLA*A0201 class I allele, such that 45% of MS patients are positive for HLA*A0201 compared to 61% of controls (odds ratio = 0.52; p = 0.005). Surprisingly, further analysis by this group indicated a protective effect of HLA*A0201, as the presence of this allele and HLA-DRB1*1501 actually lowered the risk for developing MS from 3.6 to 1.5 in a Swedish population (Fogdell-Hahn et al., 2000). Even though these associations are not the strongest, they may prove to have merit, especially when considering the fact that all cells of the CNS are capable of expressing MHC class I (Hoftberger et al., 2004; Friese and Fugger, 2005), MHC class I expression is upregulated in MS lesions (Friese and Fugger, 2005), and that the majority of infiltrating T-lymphocytes occurring in the

Table 1-1 Positive linkage regions associated with MS

Chromosome	Location
Chr1	p35, p21, q11-24, q31, q42-44
Chr2	p23, p21, p13, q24-33, 2q36
Chr3	p26, p25, p14, q21-24, 3q26
Chr4	p16, q12, q24, q26-28, q31-35
Chr5	p14-tel, **p14-12**, q11-13, q13-23, q33
Chr6	p25, **p21**, q14, q21, q22, q26, q27
Chr7	p21, p15, p14, q11, q21-22, p32-35
Chr8	8p23-21
Chr9	q21, p24-22, q34
Chr10	**p15**, p12-13, cen, q21-22, q24, q26
Chr11	p15, q25, q22
Chr12	12p13, q21, q23, q24
Chr13	q31-32, q33-34
Chr14	q32
Chr15	q21, p24-22, q34
Chr16	p13, p11, q23-24, q12
Chr17	p13, q22-24, q25
Chr18	p11, q21
Chr19	p13
Chr20	p12
Chr21	
Chr22	q12-13
ChrX	p21, p11, p22, q23-28, q26

Taken from Dyment et al., 2004 with slight modification
Highlighted regions (Chr5p13, 6p21and 10p15) represent strongest known associations with MS as discerned by Hafler et al., 2007 and encode IL-7R, MHC class II, and IL-2R respectively

lesions of MS patients are $CD8^+$ T-cells outnumbering $CD4^+$ T-cells by approximately 3:1 (Babbe et al., 2000). Additionally, there is evidence that the $CD8^+$ T-cells within MS lesions have been oligoclonally expanded (Bebbe et al., 2000). Furthermore, the number of $CD8^+$ T-cells in the CNS of MS patients has been shown to be correlative with neuronal damage (Friese and Fugger, 2005). Moreover, the negative association and protective effects of HLA*A0201 could indicate the involvement of suppressor T-

cells in curbing autoimmune $CD4^+$ T-cell responses to myelin antigens. Alternatively, this protective effect may be illustrating the benefit of this allele in clearing the CNS of an infectious agent as indicated by its ability to specifically bind epitopes from both measles and vaccinia viruses (van Els et al., 2000; Johnson et al., 2005). However, it is important to mention, that in patients with MS, HLA-A*A0201 it has been shown to recognize epitopes originating from transaldolase as well as MOG resulting in lysis pf oligodendrocytes *in vitro* (Niland et al., 2005; Mars et al., 2007).

Not long after the discovery of associations with HLA class I alleles was made, the discovery of a stronger association on the HLA class II allele was found, which may confirm the relationship with $CD4^+$-mediated responses and disease susceptibility (Jersild et al., 1973). As mentioned above, HLA-DR is highly associated with susceptibility to MS (Hafler et al., 2007). HLA-DRB1 is also strongly associated with the onset of the disease as determined through the genotyping of over 4000 individuals from Canadian and Finnish families using a high-density SNP panel, which covered the genes encoding MHC and flanking genomic regions (Lincoln et al., 2005). Within the HLA-DRB1 locus, HLA-DRB1*1501 is the most consistently associated allele (Hauser and Okenberg, 2006). However, the issue becomes more confused when other HLA alleles are taken into consideration. For instance, Okenberg recently found that HLA-DRB1*15 in the absence of DQB1*0602 is associated with the onset of MS in African American MS patients (Oksenberg et al., 2004). In contrast, Caballero et al. (1999) found the opposite, that in Brazilians of African origin DQD1*0602 and DQA1*0102 in the absence of DRB1*1501 are associated with MS onset. Even still, a dose effect for

the HLA-DR15 loci seems to exist (Barcellos et al., 2003; Modin et al., 2004). Most interesting is the relationship between HLA-DRB1*1501 (DR2b) and HLA-DRB5*0101 (DR2a), which are located 85 kb apart and are in almost complete linkage disequilibrium (always inherited together). These alleles code for MHC class II molecules that are capable of binding the immunodominant peptide MBP_{85-99} (Madsen et al., 1999). Moreover, when transgenic mice possessing these humanized alleles are crossed with $RAG^{-/-}$ transgenic mice that possess T-cells receptors (TCR) extracted from cloned T-cells from MS patients, they develop spontaneous experimental autoimmune encephalomyelitis (EAE), providing strong indirect evidence that MS is autoimmune mediated (Madsen et al., 1999). Using this "humanized mouse model" of MS, Gregersen et al. demonstrated that 88% of mice expressing human T-cell receptors from MS patients as well as DR2b, developed spontaneous EAE, while mice expressing DR2a do not develop disease (2006). Moreover, when these alleles are coexpressed, the disease incidence is decreased and the onset of EAE is delayed (Gregersen et al., 2006). Finally, using Annexin-V as a marker for apoptosis, the authors were able to conclude that DR2a functions to regulate DR2b by promoting peripheral tolerance (Gregersen et al., 2006). Taken together these results strongly demonstrate the linkage between HLA, T-cells, the immune response and MS, as well as the fact that MS can be modulated by the alleles.

Despite the strong link between HLA and MS, this region is only thought to account for 17-60% of the genetic association of the disease (Hauser and Oksenberg, 2006; Hillert, 1993; Haines et al., 1998), while the remaining 40-83% is located outside

the HLA. As the HLA is located at chromosome 6p21, this means that any of the other currently identified 76 loci on 22 chromosomes (Table 1-1) could potentially play a role in MS onset and/or exacerbation. Other than the recently observed associations with IL-2Rα and IL-7Rα (Hafler et al., 2007), some of the other candidate genes that may be involved are TCRβ chain, the inhibitory ligand cytotoxic T lymphocyte antigen-4 (CTLA-4), TNF-α, TNF-β, and the intracellular adhesion molecule (ICAM)-1 (Sospedra and Martin, 2005). Additionally, specific myelin proteins (i.e. MBP) as well as other CNS specific proteins have also been implicated of which apolipoprotein 5 (APOE4) has shown some promising, but controversial results (Sospedra and Martin, 2005).

1.7 Infectious etiology of multiple sclerosis

The possibility that multiple sclerosis could be brought on by an infectious agent was first proposed in 1884 by Charcot's former student Pierre Marie (Marie, 1884). While no infectious agent, neither viral, bacterial nor parasite, has been exclusively associated with MS onset, there is some substantial evidence that supports the role of environmental pathogens in both the onset and exacerbation of the disease. As mentioned previously, the concordance risk for monozygotic twins is approximately 30%, which means that 70% of the time the non-affected twin, sharing 100% genetic material will not develop signs or symptoms of MS, even on a subclinical level as determined by MRI (Sadovnick et al., 1993). Moreover, while 30% concurrent twin rate among monozygotic twins may support a genetic contribution to onset, it is far from causative as Cook reminds us that there was reported in 1951 a very similar concordance

rate from poliomyelitis among twins (Cook, 2006, Herndon and Jennings 1951; Eldridge, 1987).

Additionally, evidence suggestive of a pathogenic etiology consists of the previously mentioned gradient of MS prevalence. On this topic it is important to mention that migration studies have demonstrated that the risk for developing MS increases in people who migrate to a higher risk area prior to ages 15-16, whereas the risk decreases when people migrate to a lower risk area prior to ages 15-16 (Kurtzke, et al., 1985). The incidence rate does not change following onset of puberty and migration (Kurtzke, et al., 1985) which must be taken into consideration when examining the incidence among adoptees, spouses and half siblings.

Moreover, "outbreaks" of MS have been reported, with the most famous being that which occurred on the Faroe Islands (Kurtzke et al., 2001). Prior to 1943, not a single diagnosis of MS had been made on the Islands, but soon after, 76 cases were diagnosed in areas that were occupied by British troops during World War II (Kurtzke et al., 2001). As a result of these epidemiological and migrational studies, Kurtzke believes the causative agent for MS to be infectious, widespread and persistent, but asymptomatic in most cases (Kurtzke et al., 2001). He refers to this agent as "primary multiple sclerosis affection" (PMSA).

The possibility for the existence of PMSA is demonstrated by the occurrence, in animal and man, of known viruses that cause similar symptomatology to MS. For instance, canine distemper virus in dogs (Wisniewski et al., 1972), Visna virus infection in Icelandic sheep (Sigurdsson et al., 1957), Semliki Forest virus infection, the JHM

strain of mouse hepatitis virus, and Theiler's murine encephalomyelitis virus (TMEV) in mice all result in demyelination (Weiner, 1973; Daniels et al., 1952; Lipton, 1975). In man, several viral infections of the CNS are known to cause demyelination. For instance, acute disseminated encephalomyelitis, a monophasic immune-mediated demyelinating disease, is readily associated with viral infections as well as vaccinations (Tenembaum et al., 2007). Moreover, subacute sclerosing panencephalitis, another demyelinating disease, can occur within months to years following morbillivirus infection, in particular measles virus. JC virus, an endemic virus in man, can infect oligodendrocytes in severely immunosuppressed people resulting in of progressive multifocal leukoencephalopathy, a rapidly occurring demyelinating disease of which there are no known survivors (Berger, and Koralnik, 2005). Moreover, there exist widespread, generally asymptomatic latent infections with neurotropic viruses such as herpes viruses in the normal population (Challoner et al., 1995). Indeed, a great number of viruses and several bacteria have been isolated from the brains of MS patients. A list of the viruses that have been isolated from the CNS of MS patients can be viewed in Table 1-2, which demonstrates the broad spectrum of viral agents in association with

Table 1-2 Examples of viruses isolated from the CNS of MS patients

Family	Genus	Virus/Strain	Technique	Reference
Coronaviridae	Coronavirus	HCoV/229E	RT-PCR; ISH and IHC	Stewart et al., 1992; Murray et al., 1992
		HCoV/SD	HDWM and EM	Burks et al., 1980
		HCoV/SK	HDWM and EM	Burks et al., 1980
Paramyxoviridae	Paramyxovirus	Sendai Virus/(6-94)	CF	ter Meulen et al., 1972
	Morbillivirus	Measles	IHC	Geeraedts et al., 2004
		Measles	ISH	Haase et al., 1981; Cosby et al., 1989
Retroviridae	Gammaretrovirus	HERV-W (MSRV)	Endogenous; BCC	Perron et al., 1991
	Betaretrovirus	HERV-K	Endogenous	Johnston et al., 2001
	Deltaretrovirus	HTLV I	ISH[#]	Koprowski et al., 1985
Polyomavirinae	Polyomvirus	JC virus	PCR[#]	Ferrante et al., 1998
Herpesviridae	Herpesvirus	HHV-4 (Epstein-Barr virus; EBV)	ISH, IHC	Serafini et al., 2007
		HHV-6	RDA; LCMD, PCR	Challoner et al., 1995; Cermelli et al., 2003

RT-PCR = Reverse transcriptase polymerase chain reaction; ISH = in situ hybridization; IHC = immunohistochemistry;
HDWM = MS lesion homogenate at autopsy produced neurological disease after i.c. injection into mice characterized by Electron microscopy;
CF = isolation of brain cells from MS patients fused with indicator cells; BCC = isolation from MS brain cell culture; RDA = Representational difference assay;
LCMD = Laser capture microdissection

[#]Isolation of virus from cells in cerebrospinal fluid or from cerebrospinal fluid

MS. Additionally, oligoclonal bands in the CSF are known to react to viruses, and MS patients generally have increased humoral responses to viruses in their serum. MS exacerbations are also associated with viral infections (Buljevac et al., 2002). Taken together, these data may exclude the possibility that PMSA is in fact one infectious agent; rather it could be one of several agents acting within the CNS to elicit an autoimmune response. Mechanisms by which viruses could induce autoimmunity toward myelin are through molecular mimicry, bystander activation, and superantigenic activation of T-cells (myelin specific T-cell clones). The response could later be propagated to other parts of myelin by epitope spreading (Croxford et al., 2002).

Molecular mimicry occurs when a foreign antigen has a structural or molecular composition that is almost identical to that of a self-antigen resulting in autoimmunity. Currently, several autoimmune diseases are thought to have initiated from infection for which the responsible microbe contains a molecular mimic of a self antigen. Some of the most popular examples are streptococci induced Rheumatic fever, Coxsackievirus B3 infection and autoimmune myocarditis, *Borrelia burgdorfi* and Lyme arthritis, and HSV-1 and herpetic stromal keratitis (Benoist and Mathis, 2001). One of the strongest cases of molecular mimicry comes from the association of *Borrelia burgdorfi* and Lyme arthritis. *Borrelia burgdorfi*, the spirochete that causes Lyme disease, contains an epitope that closely resembles human leukocyte function-associated antigen 1α (LFA-1α; L332-340), a protein that can be isolated from synovial fluid. Interestingly, the MHC class II antigen HLA-DRB1*0401, typically associated with Lyme arthritis, is capable of binding L332-340 in Lyme arthritis patients. In conjunction, these patients

possess T-cells that respond to L332-340 (Benoist and Mathis, 2001). Taken together, these results strongly implicate molecular mimicry in the pathogenesis of Lyme arthritis.

Fairly recently, Wucherpfenning and Strominger have demonstrated how molecular mimicry could be implicated in the pathogenesis of MS patients (1995). Using L cell transfectants of DRB1*1501 (a susceptibility allele of human MS) which, as mentioned previously, is known to present a peptide sequence from myelin basic protein (MBP_{85-99}) the authors screened over 600 sequences from viral and bacterial genomes for similarity. They then tested all of the 129 potentially positive sequences for their ability to induce proliferation by co-inbubation of the L cell transfectants with human T-cell clones possessing T-cell receptors to MBP_{85-99}. Their screen demonstrated molecular mimicry to several viral sequences including herpes simplex virus, Epstein-Barr virus, adenovirus, Influenza A virus and human papillomavirus (Wucherpfennig and Strominger, 1995). More recently, Lang et al., demonstrated that the class II DRB1*1501 allele was specific for MBP_{85-99} peptide presentation, while the DRB5*0101 allele is specific for the EBV DNA polymerase sequence (molecular mimic) (Lang et al., 2002). However, a T-cell receptor isolated from an MS patient (Hy.2E11) recognized both MHC molecules (Lang et al., 2002). It is important to note that 99% of MS patients have antibodies to EBV compared to 90% of the general population; that 83-99% of children with MS are seropositive for EBV compared to 42-72% of age-matched controls, and that EBV reactivation is correlated with relapse in MS patients (Sumaya et al., 1985; Alotaibi et al., 2004; and Pohl et al., 2006; Wandinger et al., 2000). Interestingly, it has also been demonstrated that $CD4^+$ T-cell clones taken

from the CSF of patients during relapse reacted to Torque teno virus, a relatively newly discovered orphan virus (Sospedra et al., 2005). Taken together, these data demonstrate a strong potential for molecular mimicry in the pathogenesis of MS, as well as point to infectious agents as triggers for autoimmunity.

Another way in which viruses and bacteria could lead to autoimmune responses is through the expansion of autoreactive T-cell clones by superantigenic activation. While central tolerance occurring in the thymus is effective at eliminating the body of most autoreactive cells through positive and negative selection processes, some autoreactive cells escape, and are present in the circulation. This is clearly indicated by the fact that both MS patients, and healthy controls, possess T-cell clones that are myelin specific in their circulation (Frohman et al., 2006). In this regard, Sutkowski et al. have recently demonstrated that the latent membrane protein LMP-2A of EBV can transactivate the human endogenous retrovirus HERV-K18 resulting in the transcription of a superantigen (Sutkowski et al., 2001; 2004). Thus, EBV infection could theoretically bring about expansion of autoreactive T-cell clones by induction of superantigenic stimulation. Bystander activation of these clones within the CNS could also result in autoimmunity (Sospedra and Martin, 2005). Alternatively, a persistent infection may lead to the generation of new autoreactive T-cell clones by this method. In this scenario, a persistently infected tissue (especially one infected with a lytic virus) causes the constant release of self-antigens in the presence of an adjuvant (i.e. the virus). This constant inflammation may then result in the loss of tolerance (Sospedra and Martin, 2005). This concept becomes a legitimate possibility when considering that

autoimmune-mediated myocarditis is probably brought on by Coxsackievirus B3 infection (Whitton and Feuer, 2004). Moreover, Furtado et al., (2006) have shown that the conditional expression of Flt3 (an hematopoeitic factor for DCs and macrophages) in transgenic mice expressing the chemokine CCL2 in parenchymal cells resulted in spontaneous ascending paralysis that was T-cell independent (Furtado et al., 2006).

1.8 Stress and multiple sclerosis

A relatively underappreciated, but certainly prevalent, environmental stimulus that may help facilitate the onset of MS is psychological stress. Stress was first associated with MS onset by Charcot in one of his initial descriptions of the disease (Charcot, 1877). To date it has been demonstrated that nearly all MS patients report the occurrence of a major stressful life event within the two years prior to onset of their disease (Warren et al., 1982; Grant et al., 1989). Li et al. demonstrated that parents who had lost a child were at elevated risk of developing MS, and that those who lost a child unexpectedly were at even higher risk for developing MS (Li et al., 2004). Stress has also been shown to play a role in the exacerbation of RRMS, as illustrated by a meta-analysis of 14 studies conducted by Mohr et al. (2004), which showed that stress resulted in increased risk of exacerbation (Mohr et al., 2004). Most recent studies by Brown et al. indicate that it is, in fact, the number of acute stressful life events rather than chronic stressors that can predict relapse in disease (Brown et al., 2006a; Brown et al., 2006b). Mechanistically, stress could influence the pathogenesis and subsequent progression of MS in several fashions. According to Mohr and Pelletier, stress-induced dysregulation of

the hypothalamic-pituitary-adrenal (HPA) axis responses can influence MS in 3 ways: through the resolution of the stressor, by glucocorticoid resistance brought on by over stimulation of HPA axis, or by glucocorticoid induced-mast cell degranulation resulting in BBB permeability (Mohr and Pellelier, 2006).

2. Stress

2.1 Overview of stress

The effects of stress on the immune system are pronounced and are brought about directly from responses generated in the brain as a result of perceived challenges (real or not) that threaten homeostasis. The effector molecules that facilitate these responses are the hormone cortisol (corticosterone in mice) from the hypothalamic-pituitary-adrenal axis and catecholamines (epinephrine and norepinephrine) released by direct sympathetic nerve innervation of immune organs or the medulla of the adrenal gland. Interestingly, stress seems to have a bi-directional response on immunity such that acute stress can be immunostimulatory, but chronic stress is immunosuppressive (Dhabhar and McEwen, 1997), suggesting that stress (in all forms) may affect immunity to all infectious diseases.

The term "stress" is difficult to define, and as McEwen often states is ambiguous in meaning (McEwen 2005; Korte et al., 2005). However, one of the best and most precise definitions of stress is credited to Ramsey who states that stress can be defined as a state of altered (or threatened) homeostasis resulting from either an external or internal stimulus (Ramsey, 1982). Here it is important to note that this state induced by a

stressor (or stimulus) can actually be threatening, but it can also be only perceived as such by the individual. For instance, an exam (stimulus) can be perceived as threatening and become a stressor even though the exam itself does not pose much of a threat to the individual. Once the stimulus is perceived as a stressor within the brain, a physiological response will be set into motion that will attempt to deal with the altered homeostasis. Adaption to stress requires the organism to make adjustments to its allostatic properties (see below), which may include; down-regulation of glucocorticoid receptor or catacholamine receptors within the cell or changing lifestyles to address the stressor (i.e. coping mechanisms). Not all stressors or inducers of allostasis are bad, but the response to them depends on timing, duration, and type. For instance acute stressors can be healthy, but chronic stressors (ones that significantly increase allostatic load) are generally unhealthy.

Hans Selye is credited with making popular the concept of stress (McEwen, 2005). While trying to discover a new hormone, Selye noticed that his animals developed a specific response, which he termed as a syndrome, to many noxious stimuli, including cold exposure, surgical injury, morphine, formaldehyde and exercise (Selye, 1936). This response, which he coined the general alarm syndrome, consisted of three phases (Selye, 1936). The first phase, occurring 6-48 hours after an alarm response ensued, was characterized by thymic, splenic, and hepatic atrophy, decreased fat tissue, loss of muscle tone, a decrease in body temperature, and hepatic necrosis. The next response began after 48 hours and was characterized by a period of resistance. In the beginning phases of resistance, the adrenal glands were enlarged, numerous basophils

accumulated at the pituitary, general body growth stopped, and the reproductive organs ceased to function. With continued exposure to the noxious stimulus, the body's functions begin to return to normal (Selye, 1936). However, after about 30 days of exposure, the animal succumbed to the response and displayed symptoms similar to those initially seen in the first phase. Therefore, the final phase was termed "exhaustion". Together this represents Selye's general adoption syndrome (GAS) as it characterized the organisms attempt to adapt to an external challenge. Key features within this description can illustrate the stress response. First, the animal perceives a stressor and homeostasis is altered. In the second phase, responses possibly originating in the adrenal gland (as it displays hypertrophy) return the animal to a state of homeostasis. However, these responses seem to be only temporary as seen in the third phase by their failure to maintain homeostasis resulting in illness or death (McEwen, 2005).

Even though Selye's GAS model remains in many ways applicable today, several flaws in his initial concept have recently been illustrated by work from Bruce McEwen's laboratory and others. Specifically, it has been illustrated that i) the HPA and SNS work differently to control the stress response, ii) the "fight or flight" response (which is essentially the basis for Selye's model) does not equally apply to male and female responses, and iii) the exhaustion stage of Selye's model implies an overall negative connotation for stress, whereas that may not be true depending on the context in which stress occurs (i.e. immunosuppression during autoimmunity) (McEwen, 2005). Because of these reasons and the feeling that the word "homeostasis" has an overly strong

connotation in describing the influence of stress on physiological processes, McEwen and Stellar recently coined the concept of allostasis and allostatic load (McEwen and Stellar, 1993). In this concept, *homeostasis* is concerned with the maintenance of physiological set points that are essential for life, such as blood pH, body temperature, glucose levels and oxygen tension, and *allostasis* refers to the maintence of homeostatsis through the regulation of cytokines, catecholamines, and hormones of the HPA axis – those responses that are not as essential for maintaining life (McEwen, 2005). Therefore, according to McEwen (2005), one can cope with changes in allostasis inflicted by stress, and in some cases, these changes can be beneficial. For example, glucocorticoids produced from acute stress will facilitate lymphocyte trafficking from the secondary immune organs, such as the lymph nodes and spleen, to areas such as the skin in preparation for a perceived attack from a predator (Dhabhar and McEwen, 1997). However, with repeated stressor stimuli, the allostatic load increases and eventually crosses a threshold where it becomes detrimental to the animal resulting in immunosuppression (Korte et al., 2005). Factors that influence allostatic load include i) repeated challenge, ii) failure to habituate, iii) failure to shut off in the absence of stimulus and iv) failure to mount an adequate response (Korte et al., 2005).

While both Selye and McEwen illustrate models of the stress response, these models tend to differ in terms of their outcome on immunity. For example, in Selye's model, the outcome of continual stress is immunosuppression. Strict adherence to this paradigm would indicate that stress always increases the susceptibility to infectious disease and decreases in autoimmune responses. In contrast, the idea of allostasis and

allostatic load allows for differences in the outcome of infection depending on the type, duration and timing of the stressor. In this model, moderate changes in allostasis leading to increases in certain cytokines or lymphocyte trafficking may prove to be beneficial in combating infectious agents, but at the same time detrimental to patients with autoimmune diseases such as multiple sclerosis (MS). Alternatively, increases in allostatic load caused by constant stress could lead to immunosuppression, resulting in increased dissemination of a pathogen or reduction in exacerbation due to autoimmune responses.

2.2 Stress and immunity

As mentioned previously, the hypothalamic-pituitary-adrenal (HPA) axis, the sympathetic-adrenomedullary (SAM), and the sympathetic nerve innervation of the secondary lymphoid organs are directly implicated in the response to stress. In terms of the HPA axis, stress can cause increases in corticotropin releasing hormone (CRH) from the periventricular nucleus of the hypothalamus that acts on the anterior pituitary gland to release adrenocorticotropin-releasing hormone (ACTH). ACTH stimulates the adrenal cortex to secrete glucocorticoids (Webster et al., 2002). The effects of glucocorticoids are numerous and biphasic. For instance, glucocorticoids (GC) act on the liver to facilitate the release of glucose into the blood, thus regulating metabolism. However, GC elicits biphasic responses with regard to the immune system. Here, a small amount of the hormone may be beneficial (facilitating lymphocyte trafficking) whereas larger amounts are immunosuppressive. The actions of GC are facilitated in

two fashions. First, GC binds to the glucocorticoid receptor (GCR) causing dimerization, activation and its translocation to the nucleus, where it binds the glucocoroticoid response element (GRE). Binding of the GCR to the GRE will cause either the activation or repression of transcription. Alternatively the activated GCR can influence transcription by molecular "crosstalk" which involves transactivation or transrepression, mediated by direct protein to protein interactions which occur between the GCR and other transcription factors. In terms of immunosuppression, it has been shown that GCs can inflict their immunomodulatory responses at multiple levels. For instance, GCs bound to their receptor can cause the inhibition of transcription factors (including NF-kB as well as AP-1), signal transducers and activators of transcription (STATs), as well as induce the expression of genes that will also facilitate immunosuppression, and may cause an overall shift in the immune response from Th1 toward Th2 (discussed below). Thus, in high quantities GCs are highly immunosuppressive (Webster et al., 2002).

The effects of allostasis and allostatic load are also illustrated within the context of the effects of stress on the sympathetic nervous system. Here, the perception of stress will activate the SNS to release epinephrine and norepinepherine in both the adrenal medulla and the secondary lymphoid organs. The sympathetic nervous system regulates the "flight or fight" response within seconds. Following the activation of the SNS, the stomach is inhibited, the heart is stimulated, arteries constrict, and lung airways relax shifting all responses toward an alerted state (as Selye would describe it). However, unresolved stress results in arterial wall thickening due to hypertension, and adenergic

receptor desensitization (Fleshner, 2006). Like GC, the SNS can modulate the immune system. For instance, acute stress has been shown to be immunostimulatory, resulting in norepinepherine (NE) mediated enhancement of the innate immune system by releasing exogenous heat-shock protein (eHSP)72 from immune cells in an α1ADR dependent fashion (Fleshner, 2006) leading to a increased production of inflammatory cytokines, and inducible nitric oxide synthase (reviewed in Fleshner, 2006). Because these actions are independent of antigenic challenge they may indicate that acute stress has a role as an adjuvant thereby functioning as the "danger-signal" to facilitate the start of an immune response (Fleshner, 2006). These properties would be advantageous if they immediately preceded an infection, but would be disadvantageous if they occurred in patients with autoimmune diseases. Like glucocorticoids, chronic activation of the SNS results in immunosuppression. However, the mode by which this occurs is not yet clearly defined. For instance, chronic stress results in the systemic increase in catecolamines. Interestingly, Th1 and B-cells, but not Th2 cells, have receptors for catecolamines, indicating that direct exposure will modulate and perhaps shift these immune responses (reviewed in Sanders, 2006). Indeed research in this area supports the idea that increases in NE, suppress adaptive Th1 immunity. However, Fleshner has generated convincing data that suggest that a lack of NE rather than an increase in NE to the spleen is responsible for chronic stress-induced immunosuppresion of humoral responses (Fleshner, 2006). The resolution of these seemingly conflicting ideas may be attributable to the effects of NE on B-cells and Th1 cells but not on Th2 cells. Alternatively, chronic stress may result in decreased expression of enzymes from the

neuron that is responsible for the uptake of NE after secretion, thus, extending the effects of the amine. In any case, chronic activation of the SNS by stress results in the suppression of adaptive immune responses and might impede pathogenic clearance from the body but decrease exacerbation of autoimmune phenomenon.

Following viral infection, the generation of an effective immune response is summarized as follows: resident dendritic cells (DC) phagocytose the pathogen, become activated and then transport it to the nearest lymph node. Here, the antigen is presented to a helper T-cells ($CD4^+$). Upon the recognition of non-self by the T-cell and in the presence of co-activators, the T-cell will become activated, secrete cytokines which result (after recognition of the pathogen) in the activation of B-cells to secrete antibody and $CD8^+$ T-cells to kill cells infected with the pathogen. Importantly, the immune response generated by the $CD4^+$ T-cell depends on the context in which it was stimulated by the DC. For instance, T-cells stimulated with IL-12 will result in the activation and phophorylation of STAT4 resulting in activation of the transcription factor T-box-expressed in T-cells (T-bet) which is both necessary and sufficient for Th1 polarization (Szabo et al., 2000). Th1 cells are considered proinflammatory and are characterized primarily by their secretion of IFN-γ. IFN-γ can activate macrophages and DCs to upregulate MHC class II expression, as well as costimulatory molecules (CD80/CD86), increase IL-12 production, and can stimulate STAT1 in T-cells influencing T-bet expression (moreso than STAT4) providing a positive feedback loop. Th1 cells also produce Th1 type chemokine receptors (i.e. RANTES). On the other hand, IL-4 stimulation will result in the activation of STAT6 resulting in the activation of the

transcription factor GATA-3, which is necessary and sufficient for the generation of a Th2 response resulting in the secretion of IL-4, IL-5, IL-6, IL-10, and IL-13 from T-cells (Zheng and Flavell, 1997). The Th2 cell functions in humoral immunity against helminths, and is considered to be anti-inflammatory. Moreover, the chemokine CCL2 or MCP-1 and granulocyte/monocyte colony stimulating factor (GM-CSF) are considered Th2 chemokines.

Stress and glucocorticoids have profound effects on these cell signaling processes, and influence the generation of a Th1 or Th2 response. Indeed, chronic stress or GC (including the synthetic GC dexamethasone) are thought to result in a shift from Th1 immunity toward Th2 immunity (Th1/Th2 shift). However, like all other effects of stress, the timing, duration, and amount of GCs seem to play a role in these responses. For instance GC have been shown to decrease both STAT1 and STAT4 (Franchimont et al., 2000; Hu et al., 2003). Additionally, they can induce the expression of a protein called glucocorticoid-induced leucine zipper (GILZ) which can upregulate GATA-3 expression as well as STAT6 (Cannarile et al., 2006) in T-cells and is immunosuppressive in macrophages (Th2 type shift) (Berrebi et al., 2003). These results strongly suggest a GC induced Th1 to Th2 shift as one mechanism of immunosuppression toward a viral pathogen. However, it has also been shown that dexamethasone can directly inhibit the activities of GATA-3 on the IL-5 promotor (Jee et al., 2005; Quan et al., 2001). Because GATA-3 is absolutely required for Th2 polarization, these results suggest that no Th1 to Th2 shift occurs. In support of an immunosuppressive effect of stress, Dobbs et al., (1993) have demonstrated that during

experimental influenza infection in mice both virus specific Th1 and Th2 cytokines are suppressed.

Regardless of whether a Th1 to Th2 shift occurs or not, chronic stress drastically affects immune responses. In the case of immunosuppression, the host will be rendered more susceptible to pathogens, and disease will be exacerbated. In the case of autoimmunity, immunosuppression may result in alleviation of symptoms.

3. Theiler's virus

3.1 Brief history of Theiler's virus

In 1933, Max Theiler noticed that a mouse in his stock had developed spontaneous encephalitis and flaccid paralysis (Theiler, 1934). Further analysis indicated that the agent that caused this disease in mice was non-filterable and approximately the same size as that of the virus of human poliomyelitis, but shared no serological identity to human poliovirus and was not able to infect non-human primates (Theiler 1934; 1937). It was also determined early on that the natural incidence of this disease was approximately 1-2/1000 (Theiler, 1937; Sabin and Olitsky, 1938), that in mice that developed flaccid paralysis the spinal cord, but not the brain, were infectious for up to one year, and the feces infectious for up to 53 days (Theiler, 1937; Thieler and Gard, 1940). Moreover, in normal, unaffected mice the virus was present in 65% of fecal content, and when the digestive system was meticulously examined it was found that the virus was most often isolated from the wall of the small intestine (Theiler and Gard, 1940; Olitsky, 1939). These early studies indicated that the virus was endemic in

mice, was probably transmitted by a fecal-oral route, but only rarely caused disease of the CNS. Interestingly, in the early studies by Theiler, Gard, Olitsky and Sabin it was noted that the age of infection greatly influenced the subsequent pathogenesis of flaccid paralysis (Theiler, 1934; Theiler and Gard, 1940; Olitsky, 1939). In particular, mice aged 0-4 weeks often died of encephalitis, mice aged 5-7 weeks were most readily affected, but mice infected at a later age were completely resistant to the paralysis and disease (Theiler, 1934; Theiler and Gard, 1940; Olitsky, 1939).

Because of the size of the virus, and the appearance of polioencephalitic disease following intracerebral inoculation, the "virus of spontaneous encephalitis of mice" or Theiler's virus, as it became known, was used as a mouse model of human poliomyelitis. However, subsequent histological and immunological characterization of the disease caused by Theiler's virus would eventually change the use of TMEV from a model of human poliomyelitis to a model for human multiple sclerosis. The first findings that began this process occurred in 1948 when Joan Daniels recovered a strain of virus from her mouse colony at Harvard. After extensive characterization of this virus, which was determined to be a strain of Theiler's virus, the results were published in 1952. It was in this paper that Joan Daniels first described a chronic form of disease which was characterized by demyelination (Daniels et al., 1952). According to Daniels multiple lesions occurred in the meninges, the brain (medulla, midbrain, cortex, hippocampus and cerebellum), spinal cord, anterior nerve roots and sometimes in the peripheral nerves following intracerebral inoculation, but less frequently following intraperitoneal inoculation (Daniels et al., 1952). As these lesions were similar to those induced with

spinal cord homogenate in complete freunds adjuvant in mice as described by Rain et al., (Experimental allergic encephalomyelitis) the authors could not rule out the possibility of post vaccination encephalomyelitis as their inoculate was generated from dilutions of spinal cord homogenate (Rivers et al., 1933).

In 1975 the issue of pathogenesis was resolved. By using Daniel's (DA) strain of Theiler's virus adapted to grow in baby hamster kidney (BHK) cells, Howard Lipton described a biphasic disease consisting of an early encephalitic phase followed by a chronic progressive phase in which 84% (n=177) of infected Swiss mice demonstrated demyelination (Lipton, 1975). Like Daniel et al. (1952), Lipton also described an inflammatory infiltrate at the lesion site, an occurrence that has similarities to human MS (Lipton, 1975). Subsequent studies indicated that the demyelination process occurring in the chronic disease state was immune-mediated as it could be inhibited by various immunosuppressive agents including administration of cyclophosphamide or anti-thymocyte antibody (Lipton and Dal Canto, 1976) as well as by the administration of anti-CD4 or anti-CD8 antibodies (Welsh et al., 1987). Importantly, because a tissue adaptive strain of TMEV was used, the inoculate contained no residual CNS tissue and Lipton was able to dispel the possibility that demyelination was attributable to a post-vaccination encephalomyelitis (Lipton, 1975). Figure 1-1 demonstrates the differences between acute encephalomyelitis, which occurs during early disease (Fig. 1-1A-D), and the immune-mediated chronic demyelination phase which occurs in late disease (Fig. 1-1E-H), confirming Lipton's initial findings.

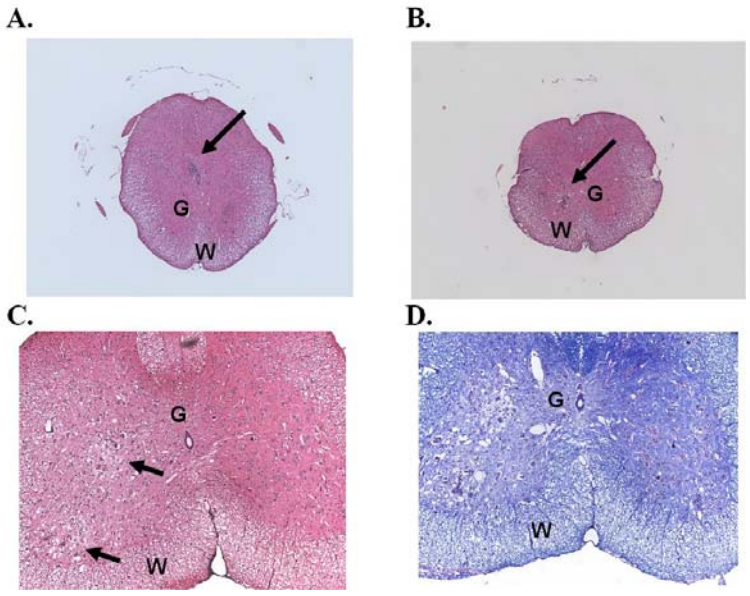


Figure 1-1. Biphasic nature of Theiler's virus infection. A-D. Male SJL mouse experimentally infected with the BeAn 8386 strain of TMEV spontaneously presented with complete paralysis of the left leg accompanied by paresis of the contralateral limb as well as encephalitis at day 31 p.i. A. H&E staining shows focal inflammation of the gray matter lateral to the central canal (arrows). B. Serial sections moving caudally, indicated focal inflammation of the lower motor horn of the gray (G) matter (arrows). C. Lumber sections showed diffuse inflammation with tissue destruction (arrows) corresponding to the ipsilateral side of paralysis . D. Luxol fast blue staining demonstrated complete sparing of the white mater (W). E-H. Male SJL mouse showing typical signs of chronic demyelination sacrificed at day 195 p.i. E. Diffuse inflammation of the white (W) matter in the thoracic section of the spinal cord is illustrated by H&E staining, while serial sections stained for myelin with luxol fast blue (F) demonstrated two separate demyelinating lesions (arrows). G. Perivascular cuffs confined to the white matter (W) are easily distinguishable (arrows) by H&E staining which typically surround active sites of demyelination (H) illustrated by luxol fast blue staining.

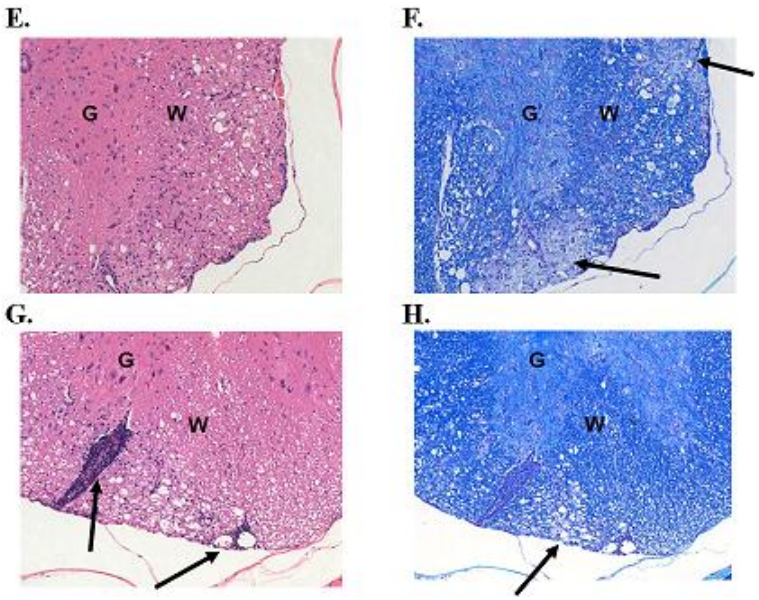


Figure 1-1, continued.

Because infection with TMEV causes immune-mediated demyelination, the production of anti-myelin antibodies in the serum (Rauch et al., 1987), including antibodies to MBP, MOG_{35-55}, and $PLP_{139-151}$ (Sieve et al., 2004), intrathecally produced antibodies (Pachner et al., 2007a), is pathologically influenced by gender (males affected more severely than females) (Fuller et al., 2005), the H-2 and non-H-2 associated alleles (Brahic et al., 2005), and the prevalence of virus among mice made this an excellent model in the study of human MS (Olesack et al., 2004).

3.2 Theiler's virus infection and replication

Theiler's viruses belong to the *Picornaviridae* family of viruses and the genus cardiovirus (Ozden et al., 1986; Pevear et al., 1987). Like other members of the family, including poliovirus, coxsackievirus, rhinovirus, foot and mouth disease virus,

encephalomyocarditis, and mengo virus, the Theiler's viruses are small in size (28-30nm in diameter), contain a pseudo T=3 icosohedron shape, and consist of a positive sense single stranded RNA genome which is approximately 8100 neucleotides in length and contains one open reading frame. Upon entry into the cell, (i.e. macrophages, monocytes, microglia, cerebrovascular endothelial cells, astrocytes, neurons, intestinal epithelial cells ect.) for which the receptor is not yet known, Theiler's replication is thought to proceed in a fashion that is not dissimilar to other picornaviruses. Here, the RNA genome, stabilized by the binding of the VPg to the poly-A tail template, contains an intraribosomal entry sequence (IRES) within the leader sequence. This secondary RNA structure is able to bind to the ribosomes and is translated into a single long polyprotein. This protein is subsequently cleaved into three progenitor proteins termed P1, P2, and P3. The progenitor proteins undergo further cleavage processes which result in capsid proteins (VP1, VP2, VP3, VP4) from P1 and proteins involved in replication or cleavage from P2 (2A, 2B, 2C) and P3 (3A, 3B, 3C, 3D) for a total of 12 proteins. The exception is the generation of L* which is translated from a sub-genomic reading frame located within the leader strand of both DA and BeAn 8386 strains (Oleszack et al., 2004; Brahic et al., 2005). As members of the family *Picornaviridae* do not contain an intravirion RNA dependent-RNA polymerase, replication of the genome follows protein synthesis, which both occur in the cytoplasm. Indeed, protein 3D is the RNA-dependent-RNA polymerase for TMEV (Johnston et al., 1995) as well as other picornaviruses. Following the generation of 3D, replication of the genome occurs from a 5' to 3' fashion within the cytoplasm producing a negative sense genomic strand, and

potentially a double stranded RNA intermediate. The negative strand is used again as a template by the RNA-dependent-RNA polymerase and transcribed back into a positive sense strand. The genome is packaged into the virion and the marture virions are released from the cell by lysis.

Interestingly, while sequences across strains of Theiler's viruses share 90.4% homology (Pevear et al., 1988) these strains can be further classified by pathology. For instance, GDVII and FA are extremely neurovirulent and result in rapid onset of paralysis and fatality following nearly all routes of inoculation due, at least in part, to encephalitis (Kohanawa et al., 1995; Villarreal et al., 2006). On the other hand, the Theiler's Original (TO) subtypes which consists of the DA, BeAn 8386, Yale, and WW strains, are less neurovirulent, typically resulting in a sub-clinical acute infection which is easily cleared in resistant strains of mice. However, in susceptible strains of mice, the TO viral strains will persist in glia (microglia, astrocytes, oligodendrocytes), and macrophages and monocytes for the duration of the animal's life-span, and contribute to the onset and progression of the previously mentioned immune-mediated demyelination process.

3.3 Innate immune responses to TMEV infection

Clearance of viral infection is dependent on the generation of adaptive immune responses. Innate immunity, acting through natural killer (NK) cells, activated antigen presenting cells (APC), and various cytokines and chemokines plays an imperative role

in both the limitation of viral dissemination and replication, as well as the generation of virus-specific adaptive immune responses during acute infection (Kim et al., 2005).

The function of the innate immune response to TMEV infection has been the subject of considerable study and has recently been reviewed (Kim et al., 2005). For instance Paya et al., (1989), as well as others, have demonstrated ablation of the NK cell response, through antibody-mediated depletion or through the use of NK cell deficient mice, resulted in increased gray matter disease of the central nervous system (Paya et al., 1989; Kim et al., 2005). Furthermore, because NK cells are an important source of IFN-γ, which can act to facilitate the signal transducer and activator of transcription (STAT)1 dependent activation of the transcription factor T-box expressed in T cell (T-bet), early decreases in NK cell activity would be expected to influence Th1 polarization (Szabo et al., 2000). Another role for NK cells in aiding in resistance is supported by studies that demonstrated that IFN-γ was required for resistance to TMEV-induced demyelination (Rodriguez et al., 1995). It is important to note that while it has been shown that both $CD4^+$ and $CD8^+$ T-cells are important sources of this cytokine, the role of NK cells as a source of IFN-γ has not completely been elucidated (Murray et al., 2002).

Other cytokines have also been shown to be induced by infection with TMEV (Kim et al., 2005). Of particular interest is the association of IL-12 with TMEV pathogenesis. IL-12 is a heterodimer composed of IL-12(p35) and IL-12(p40) subunits forming active IL-12(p70). Active IL-12, signaling through STAT4, strongly induces Th1-mediated responses in T-cells. Interestingly, the depletion of IL-12 by antibody administration during the acute phase of TMEV infection delays the onset of

demyelination in susceptible mice (Inoue et al., 1998), whereas STAT4-/- mice on a normally resistant background are made susceptible to demyelination (Rodriguez et al., 2006). Moreover, it has recently been suggested that IL-12(p70) may limit infectivity of macrophages, the cell lineage in which the virus is thought to persist, resulting in the differences that are observed in susceptibility between SJL and B.10S strains of mice (Petro, 2005a).

Chemokines are small proteins that function in the chemoattraction of immune cells, including T cells, to the site of infection thus aiding in viral clearance. Theiler's virus infection causes a marked increase in chemokine mRNA expression in the CNS of both susceptible and resistant strains of mice (Ransohoff et al., 2002; Mi et al., 2004; Kim et al., 2005), and this increase has been shown to correspond with decreases in viral titers. However, in the susceptible SJL strain, the chemokines RANTES and MCP-1 remain elevated during later disease, and likely contribute to Theiler's virus induced demyelinating disease (TMEV-IDD) pathogenesis (Ransohoff et al., 2002). Additionally, the chemokine MIP-2 has also been associated with TMEV-IDD (Rubio et al., 2006). Nevertheless, chemokine expression during acute infection is viewed as immunoprotective, as it aids in viral clearance through the recruitment of immune cells to the site of infection.

3.4 Adaptive immune responses to TMEV infection

While the degree of susceptibility to TMEV-IDD may be multigenic, resistance to this disease has been mapped to the H-2D locus (Brahic et al., 2005) such that mice of

the haplotypes H-2D^{d,b,k} haplotypes are resistant, whereas mice of the H-2D^{s,v,q,r,f} haplotypes are susceptible (Azoulay-Cayla et al., 2000; Rodriguez and David 1985). This association of resistance with class 1 antigen haplotypes implies the association of CD8^{+} T-cells in viral clearance. Findings in support of this hypothesis are (i) deletion of CD8^{+} cells by antibody administration exacerbates disease (Borrow et al., 1992), (ii) β-2 microglobulin deficient (Begolka et al., 2001; Fiette et al., 1993; Miller et al., 1995; Pullen et al., 1993; Rodriguez et al., 1993), (iii) perforin deficient (Palma et al., 2001; Rossi et al., 1998; Murray et al., 1998) and (iv), CD8$^{-/-}$ (Ransohoff et al., 2002) mice generated on a Theiler's virus-induced demyelination resistant background are rendered susceptible. Additionally, it has been shown that an early and robust cytotoxic response (Dethlefs et al., 1997a) to a single immunodominant epitope (VP2$_{121-159}$) is sufficient for viral clearance and resistance (Dethlefs et al., 1997b; Mendez-Fernandez et al., 2003). Recently, Kang et al. (2002a) have demonstrated that susceptible SJL mice are capable of mounting a robust response to the BeAn epitopes VP3$_{159-166}$, VP3$_{171-190}$ and VP1$_{11-30}$ with the response to VP3$_{159-166}$ predominating (Kang et al., 2002a), but quantitatively this response occurring within the CNS is not sufficient to prevent viral persistence (Lyman et al., 2004). Interestingly, another study conducted by the same group involving susceptible SJL mice and the comparison of the CD8^{+} T-cell response directed against the BeAn and more virulent DA strain of TMEV, suggested that the more severe outcome following DA infection maybe due to the fact that DA has only one immunoreactive epitope compared to the three that occur within the BeAn strain (Kang

et al., 2002b). The known immunodominant $CD8^+$ T-cell restricted epitopes are shown in Table 1-3.

The generation of a strong antiviral $CD8^+$ T-cell response is subsequent to the formation of a Th1 type CD4 T-cell-mediated response. The protective role of $CD4^+$ T-cells in the acute phase of TMEV infection is undisputedly illustrated by the survival curves that can be generated in both susceptible and resistant mice in their absence (Welsh et al., 1987; Ransohoff et al., 2002). Indeed, the depletion of $CD4^+$ T-cells by either genetic manipulation (Ransohoff et al., 2002) or thymectomy coupled with intravenous administration of anti-CD4 antibodies has been shown to result in high mortality rates for C57BL/6, PL/J (40 and 60% respectively by day 6 post infection), and CBA (40% by day 20 post infection) mice (Welsh et al., 1987; Ransohoff et al., 2002). Alternatively, prior immunization with the TMEV-specific immunodominant $CD4^+$ peptides in complete Freund's adjuvant delays the onset of TMEV-induced demyelinating disease (Mohindru et al., 2006). The immunodominant $CD4^+$ T-cell restricted epitopes are shown in Table 1-4. Interestingly, while prior immunization with these epitopes is protective, continued immune responsiveness to some of these, particularly $VP2_{74\text{-}86,}$ correlates with demyelination during chronic infection (Gerety et al., 1991; 1994).

Table 1-3 Known immunodominant $CD8^+$ T-cell epitopes for TMEV

Mouse Strain	Virus Strain	Epitope	Restriction	Comment	Reference
	DA	$VP2_{121-130}$	$H\text{-}2D^b$	Confers Resistance	Mendez-Fernandez et al., 2003
C57BL/6	BeAn	$VP2_{121-130}$	$H\text{-}2D^b$	Dominant; Tc1	Lyman et al., 2002
	BeAn	$VP3_{101-120}$	$H\text{-}2K^b$, $H\text{-}2D^b$	Sub-Dominant;Tc2	Lyman et al., 2002
	BeAn	$VP2_{161-180}$	$H\text{-}2K^b$, $H\text{-}2D^b$	Sub-Dominant;Tc2	Lyman et al., 2002
	DA	$VP1_{11-20}$	$H\text{-}2K^s$	Dominant; No Sub-Dominant	Kang et al., 2002a
SJL	BeAn	$VP3_{159-166}$	$H\text{-}2K^s$	Dominant; IFN-γ Secreting	Kang et al., 2002b
	BeAn	$VP1_{11-20}$	$H\text{-}2K^s$	Sub-Dominant; IFN-γ Secreting	Kang et al., 2002b
	BeAn	$VP3_{173-181}$	$H\text{-}2K^s$	Sub-Dominant; IFN-γ Secreting	Kang et al., 2002b

Table 1-4 Known immunodominant $CD4^+$ T-cell epitopes for TMEV

Mouse Strain	Virus Strain	Epitope	Restriction	Comment	Reference
C57BL/6	BeAn	$VP4_{25-38}$	?	IFN-γ Secreting	Kang et al., 2005
	BeAn	$VP2_{206-220}$	?	IFN-γ Secreting	Kang et al., 2005
SJL	BeAn	$VP2_{74-86}$	H-2I-A^s	Dominant; Role in Pathogenesis	Gerety et al., 1991; Gerety et al., 1994
	BeAn	$VP1_{233–250}$	H-2I-A^s		Yauch and Kim, 1994
	BeAn	$VP3_{24–37}$	H-2I-A^s	Proliferative	Yauch et al., 1995

Antiviral antibodies become detectable in the serum of infected mice beginning as early as day 8 p.i., and increase during the course of infection. Moreover, Pachner et al. (2007a; 2007b) have recently demonstrated intrathecal antibody production, as well as antibody in the CSF during the course of TMEV infection, with both the BeAn 8386 and DA strains. Using ELISPOT, RT-PCR and ELISA techniques, the authors were able to demonstrate a logarithmic humoral response occurring within the CNS which begins as early as 30 days p.i. and correlates highly with viral load (Pachner et al., 2007a). It is believed that antiviral antibody responses to TMEV aid in viral clearance through neutralization. For example, Kang et al., (2005) showed that antibody-mediated neutralization of virus contributed to resistance even in the absence of a $CD8^+$ T-cell response (Kang et al., 2005). Additional evidence supporting a role for anti-viral antibody-mediated decreases in pathology is that rising antibody titers correlated with decreases in viral titers (Welsh et al., 1989), and that gender differences in susceptibility are accounted for by anti-viral antibody responses (Fuller et al., 2005). Furthermore, as illustrated with the $CD4^+$ response, immunization with the B-cell immunodominant epitope prior to infection conveys slight protection in susceptible mice (Oleszak et al., 2004).

3.5 Stress and TMEV infection

To date we have shown that chronic stress, brought on by physical restraint, activates the HPA axis of CBA and SJL mice as demonstrated by increased adrenal mass coupled with increased serum corticosterone levels (Campbell et al., 2001; Sieve et al.,

2004). In turn, HPA activation causes a decrease in body, splenic and thymic mass as well as a reduction in total blood lymphocytes (Campbell et al., 2001; Welsh et al., 2004). As early as day 2 p.i. with TMEV, RS has been shown to decrease mRNA expression of the chemokines IP-10, Ltn, MCP-1, RANTES, and TCA-3 in the spleens of CBA mice as determined by RNA protection assay (RPA; Mi et al., 2004). By day 7 p.i. the virus-induced mRNA expression of the chemokines IP-10, RANTES, and Ltn in the brain are reduced by stress (RPA; Mi et al., 2004). Decreases in these chemokine mRNA expression levels, particularly in the brain, seem to correspond to decreased inflammatory infiltrates in the brain, implying that restraint stress alters cell trafficking to this organ (Mi et al., 2004). In male CBA mice, two or three sessions of RS was found to increase TNF-α levels while two sessions of RS were found to decrease the TMEV-specific NK cell response (Welsh et al., 2004). Taken together, these findings indicate an overall negative effect of stress on the immune function which is most clearly demonstrated by the finding that stressed mice have increased viral titers in most all organs (Mi et al., 2006b) compared to non-stressed mice including in the central nervous system (Campbell et al., 2001).

While a plethora of information has been generated on the effects of restraint stress in the acute phases of Theiler's virus infection (Campbell et al., 2001; Mi et al., 2004; Welsh et al., 2004; Mi et al., 2006a; 2006b) not many studies have addressed the effects of chronic stress in the acute phase of Theiler's virus on the outcome of the demyelinating phase of Theiler's virus. However, Sieve et al, have shown that as a result of RS, immune impairment in the early phase of TMEV infection coupled with

unchecked viral replication seems to lead to an earlier and exacerbated demyelinating disease as evidenced both clinical and histological findings (Sieve et al., 2004; Sieve et al., 2006).

II RESTRAINT STRESS-INDUCED DECREASES IN NK CELL ACTIVITY CORRELATE WITH INCREASED VIRAL TITERS DURING ACUTE THEILER'S VIRUS INFECTION

1. Introduction

Theiler's murine encephalomyelitis virus (TMEV) infection with either DA or the BeAn strain provides a unique model for the study of virally-induced demyelination. Additionally, its clinical, histological and immunological similarities to secondary progressive multiple sclerosis (MS), makes TMEV infection a good model for the study of human MS (Lipton, 1975). Following intracranial (i.c.) injection with either DA or BeAn strains of TMEV, susceptible strains of mice (e.g. SJL and CBA) develop acute gray matter disease followed by chronic inflammation of the central nervous system (CNS) white matter, leading to immune-mediated demyelination (Oleszak et al., 2004). The ensuing chronic demyelinating disease is dependent on viral persistence within the CNS (Brahic et al., 1981). In contrast, resistant mice, through a well-orchestrated immune response, are able to clear the virus early and, as a result, do not develop the chronic demyelinating form of the disease (Brahic et al., 2005).

Interestingly, it has been reported that as many as 77% of MS patients experienced a major stressful life event prior to disease onset (Grant et al., 1989). Psychological stress has been shown to modulate the immune system through the hypothalamic-pituitary-adrenal (HPA) axis, as well as through the autonomic nervous system (reviewed by Webster et al., 2002). Generally, the effects of stress are

determined by both the duration and type of stressor (Dhabhar and McEwen, 1997). In terms of chronic stress, immunosuppression is achieved by glucocorticoid-induced apoptosis, decreased cell trafficking, and an overall Th1 to Th2 shift (Webster et al., 2002).

We have demonstrated that stress, during acute TMEV infection, results in a decrease in mRNA expression of chemokines and cytokines (Mi et al., 2004 and 2006a), a decrease in NK cell cytotoxicity (Welsh et al., 2004), and splenic and thymic atrophy (Campbell et al., 2001) in CBA mice. In our model, immunosuppression in the acute phase of TMEV infection by RS, results in higher CNS viral titers (Campbell et al., 2001; Mi et al., 2006b) which in turn causes an earlier onset of the demyelinating phase, as well as increased disease severity in SJL mice (Borrow et al., 1992; Sieve et al., 2004).

While the adaptive immune response to TMEV is ultimately responsible for the clearance of the virus from the CNS, the innate immune response may play an important role in limiting early viral replication. Even though natural killer (NK) cells are not directly responsible for conferring resistance to TMEV (Paya et al., 1989; Lin et al., 2003), their depletion via monoclonal antibody administration has resulted in increased gray matter inflammation following DA infection of C57BL/6 mice (Paya et al., 1989). In TMEV infection, susceptible SJL mice were found to have a 50% lower NK cell activity compared to resistant C57BL/6 mice (Paya et al., 1989). The low activity of NK cells in the SJL mice is due to a differentiation defect in the thymus that impairs the responsiveness of NK cells to stimulation by IFN-β (Kaminsky et al., 1987). In

addition to lysing virally-infected cells, NK cells have the ability to secrete IFN-γ, a potent Th1 cytokine that plays a major role in TMEV clearance (Pullen et al., 1994; Rodriguez et al., 1995; Murray et al., 2002; Rodriguez et al., 2003). We and others have shown that RS can decrease virally-induced NK cell activity (Welsh et al., 2004; Tseng et al., 2005).

Here, we were interested in the effects of RS on NK cell activity at day one post infection in three strains of mice of varying susceptibility to Theiler's virus. Using BALB/c, CBA and SJL mice we investigated NK cell activity using cytotoxicity, cytokine production and viral titers in the blood, and brain. We hypothesized that NK cell responses would correlate with the degree of susceptibility in these strains, and that stress would lead to increased viral titers in all strains.

2. Methods and materials

2.1 Mice

Three to four-week old female SJL, CBA and BALB/c mice were purchased from Harlan (Indianapolis, IL). Upon arrival, the mice were counterbalanced according to strain by weight and assigned to either the infected restrained (I/R), infected non-restrained (I/NR), non-infected restrained (NI/R) or non-infected non-restrained (NI/NR) groups. Each mouse was identifiable by unique cage number and markings on made on the tale. Mice were housed five per cage. All mice were given one week to acclimate to their housing environment (light dark cycle 500 to 1700) prior to the onset of the

experiment. Throughout the duration of the experiment food and water, provided by the animal care facility at Texas A&M University were provided *ad libitum*.

2.2 Virus

The BeAn strain of Theiler's virus (kindly provided by Dr. H. L. Lipton, Department of Microbiology-Immunology, University of Illinois at Chicago, IL) was initially propagated in lung tumor (L2) cells (Welsh et al., 1987).

2.3 Infection and restraint

All mice in the infection groups were anesthetized with isoflurane (Vedco, MO, USA) and immediately inoculated in the right cerebral hemisphere with 5.0×10^5 pfu BeAn in 20µl of media. Non-infected groups were inoculated in the right cerebral hemisphere with 20µl sterile PBS (pH 7.2). Mice in the restraint groups (I/R and NI/R) were subjected to eight hours of restraint stress (from 1200 to 800) one night prior to infection and one night following infection for a total of two nights. During each restraint session, mice were placed in well-ventilated 60 ml syringes as described previously (Sieve et al., 2004).

2.4 Body mass

Total body mass was recorded for each mouse following the first session of restraint (day 0 pi) and at the conclusion of the experiment (day 1 pi).

2.5 Tissue isolation

At day 1pi, mice were sacrificed with 150mg/kg beuthanasia-D special (Schering-Plough Animal Health). Blood was collected from all mice via transection of the subclavian artery. The brains were extracted and placed in 2.0ml cryogenic vials (Corning Inc. New York, USA), flash frozen in liquid nitrogen and stored at -80°C until used for plaque assays. Serum was obtained by centrifugation of clotted blood at 3000 x g for 10 minutes and was stored at -80°C until used to assay for cytokine profiles. In addition, the cellular fraction of whole blood including red blood cells was retained, and kept at -80°C until it was used for plaque assays. Single cells suspensions were made from the spleens as described previously (Welsh et al., 2004).

2.6 NK cell activity

NK cell activity was assessed using a standard ^{51}Cr release assay as previously described using splenocytes as effectors and YAC cells as targets (Welsh et al., 2004). Total ^{51}Cr release was determined by incubating targets with 10% Triton-X 100. Spontaneous release was determined by counting the amount of ^{51}Cr released after incubation of targets in complete RPMI media. Total lysis of experimental samples was calculated using the following equation: [(experimental release – spontaneous release)/(total release – spontaneous release)] x 100. All samples were run in triplicate.

2.7 Plaque assays

Plaque assays were used to determine the amount of virus present in brains, and in the cellular fraction of blood as described previously (Welsh et al., 1987).

2.8 Cytokine profiling

Serum IFN-γ and TNF-α levels were determined using bioplex kits according to the manufacturer's instructions (Bio-rad Laboratories Hercules, CA). Positive control serum for this assay was generated by the intraperitoneal injection of an SJL mouse with a sub-lethal dose (100μg/mouse) of lipopolysaccharide (LPS) (Sigma). After 24 hours, the mouse was sacrificed, blood collected, and the serum stored at –80° C until cytokine levels were determined.

2.9 Statistical analysis

Data are presented as mean ± SEM. Correlations were assessed using simple regressions. All other data were analyzed using analysis of variance (ANOVA). When appropriate, Fisher's protected least significant difference model was used for post hoc comparison of means. In all cases significance was set at $\underline{p} \leq 0.05$.

3. Results

3.1 Both restraint stress and TMEV infection had marked effects on body mass

We found that restraint decreased body mass in infected and non-infected CBA, SJL and BALB/c mice ($\underline{p} < 0.001$; Fig. 2-1). In addition, infection alone was also found

to significantly decrease body mass at day 1 p.i. in all strains tested ($p < 0.001$; Fig. 2-1), and there was a strong trend supporting an interaction between restraint stress and infection but this trend was not significant ($p = 0.0514$).

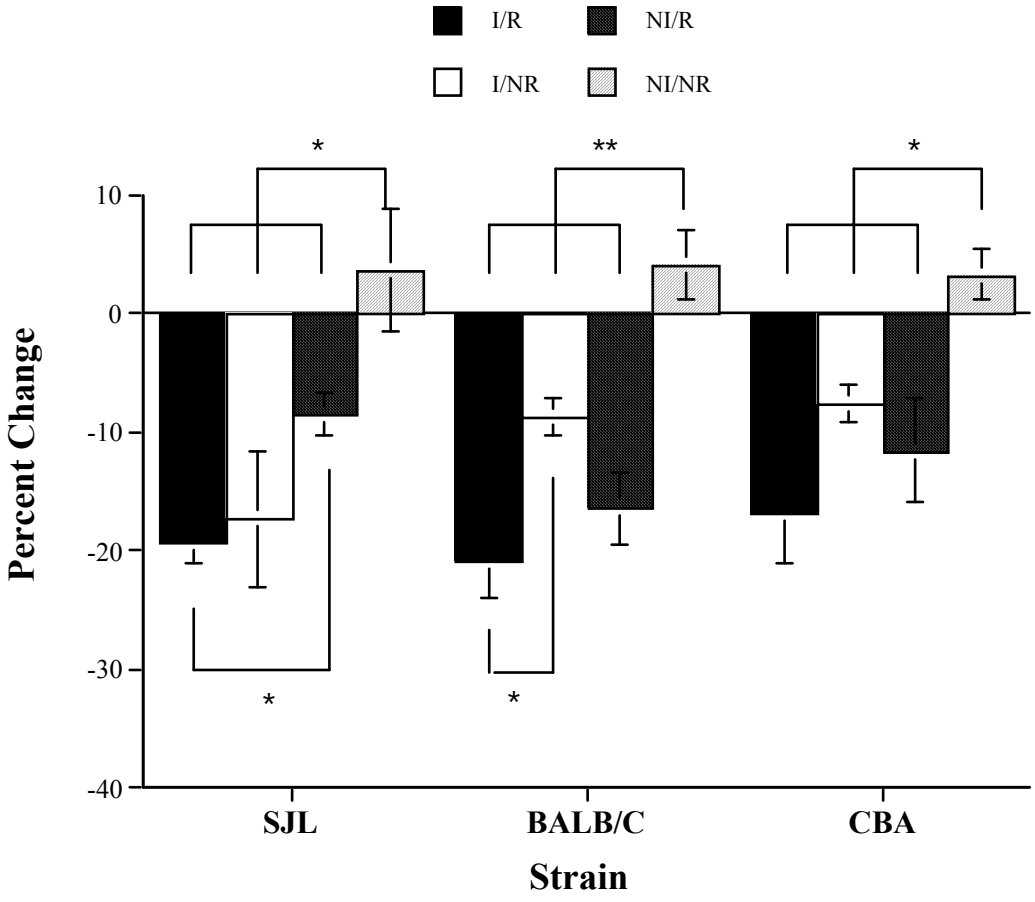


Figure 2-1. The effects of infection and restraint stress on body mass. Body mass was recorded prior to infection with the BeAn strain of TMEV, and at 1 day post infection the percentage change in body mass was calculated for all three strains of mice. In all strains, mice in both the restraint (NI/R, I/R) and infected (I/NR, I/R) groups showed marked decreases in body mass. Results are expressed as means ± SEMs. *$P < 0.05$, ** $P < 0.01$.

3.2 Effects of restraint stress and TMEV infection on IFN-γ and TNF-α levels

Serum IFN-γ and TNF-α levels were measured using luminex technology with an IFN-γ and TNF-α kit (Bio-rad Laboratories Hercules, CA). IFN-γ levels were found to be consistently below the detection levels of the assay (< 0.2 pg/ml). No differences were found in serum TNF-α levels between groups within strains, although it appeared that BALB/c mice possessed on average higher serum TNF-α levels than the other strains (Fig. 2-2A). Therefore, for each strain of mouse the groups were averaged to determine if differences existed between mouse strains. There was a significant strain difference in serum TNF-α levels, ($p < 0.05$), such that BALB/c mice had higher levels than either CBA or SJL (confirmed by Post hoc $p < 0.05$) (Fig. 2-2B). Serum TNF-α levels were identical between CBA and SJL mice. No significant differences were found to be attributable to either restraint stress or infection in TNF-α levels. To ensure the validitiy of our findings, serum from a SJL mouse previously injected 24 hours prior with LPS was included in the assay as a positive control (Fig. 2-2B).

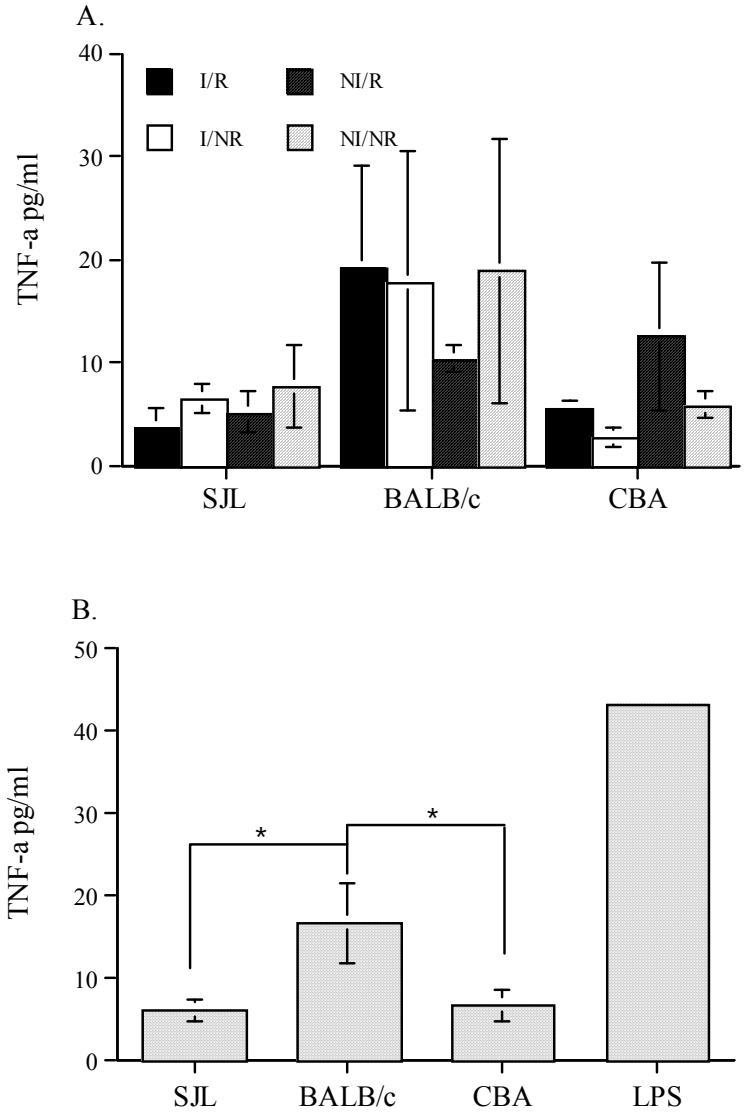


Figure 2-2. The effect of stress and strain on serum TNF-α levels. Serum cytokine levels were measured using a IFN-γ and TNF-α kit. A. Effects of stress on goups within strains. B. BALB/c mice had higher basal TNF-α levels than SJL or CBA mice. Serum from a single LPS injected SJL mouse was included as a positive control and was run in douplicate. Results are expressed as mean ± SEMs. * $P < 0.05$.

3.3 Effects of restraint stress and TMEV infection on splenic NK cell lysis

Doubling dilutions of effector to target ratios revealed the specificity of the NK cell cytotoxcity assay (Fig. 2-3A) and, an effector to target ratio of 100:1 was used for all comparisons. Infection with TMEV resulted in an overall increase in NK cell activity ($p < 0.01$). Conversely, restraint stress significantly decreased overall NK cell activity ($p < 0.001$). Interestingly, NK cell percent lysis was found to be significantly different between strains ($p < 0.001$), such that CBA > BALB/c > SJL (confirmed by Post hoc all ps ≤.0054) (Fig. 2-3B). In addition there were significant interactions noted between both strain and infection ($p < 0.05$) as well as strain and stress ($p < 0.05$). However, the interaction between infection and stress failed to reach significance (Fig. 2-3B). Analyses between individual groups reflected the above mentioned interactions. For instance, in CBA mice, the I/NR group had the highest percent lysis, which was reduced in I/R mice ($p < 0.05$). Additionally, in CBA mice the level of percent lysis was found to be reduced in NI/R mice when compared to NI/NR mice (confimed by Post hoc, all ps < 0.05; Fig. 2-3B). Alternatively, in BALB/c mice the I/NR group had significantly higher levels of pecent lysis than NI/R mice ($p < 0.001$), but were not found to differ from those of I/R mice ($p = 0.063$). In addition, the levels of percent lysis did not differ between NI/NR mice and NI/R mice ($p = 0.077$). In order to fully understand the significant interactions between strain and infection as well as strain and stress, 2-way ANOVA's were conducted on each individual strain. These subsequent analyses confirmed the previous findings for both BALB/c and CBA mice (Fig. 2-3B). However, the splenic NK cell response in SJL mice was neither influenced by stress nor infection

(Fig. 2-3B). This was probably due to the fact that NK cell responses in this strain are defective (Kaminsky et al., 1987).

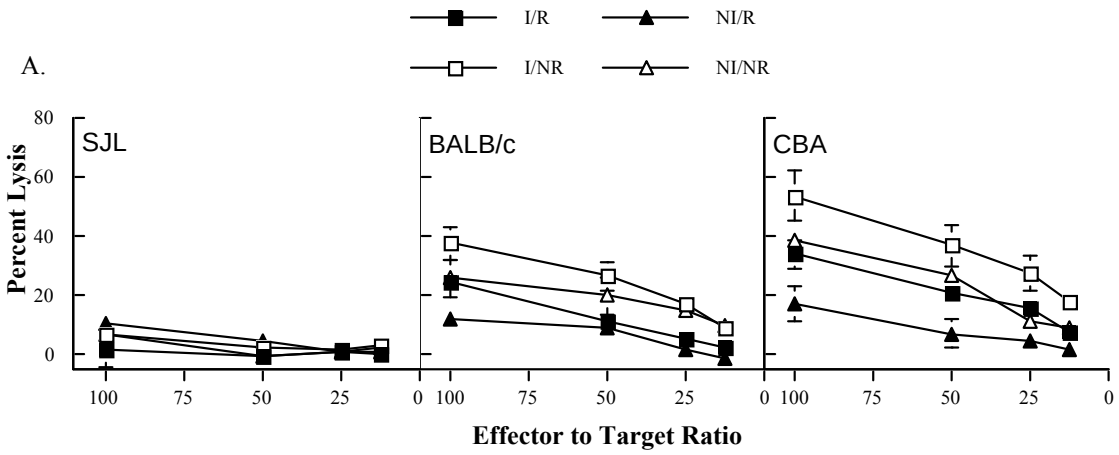


Figure 2-3. Infection and restraint stress influenced NK cell lysis in CBA and BALB/c mice. A. The effects of infection and restraint stress on NK cell lysis at day 1 post infection with TMEV were determined by a standard ^{51}Cr release assay using YAC-1 cells as targets and doubling dilutions of splenocytes as effectors. B. Infection increased NK cell activity in CBA but not BALB/c mice while restraint stress decreased NK cell activity in these strains. Neither infection nor restraint influenced the NK cell activity in SJL mice. CBA mice generated the strongest splenic NK cell response, BALB/c an intermediary response and SJL the lowest response. C. Conversely, restraint stress increased the viral load in BALB/c and CBA mice. The brains of SJL mice appeared to have higher levels of TMEV than BALB/c or CBA, but this effect did not reach significance. Results are expressed as means ± SEMs. * $P < 0.05$, ** $P < 0.01$.

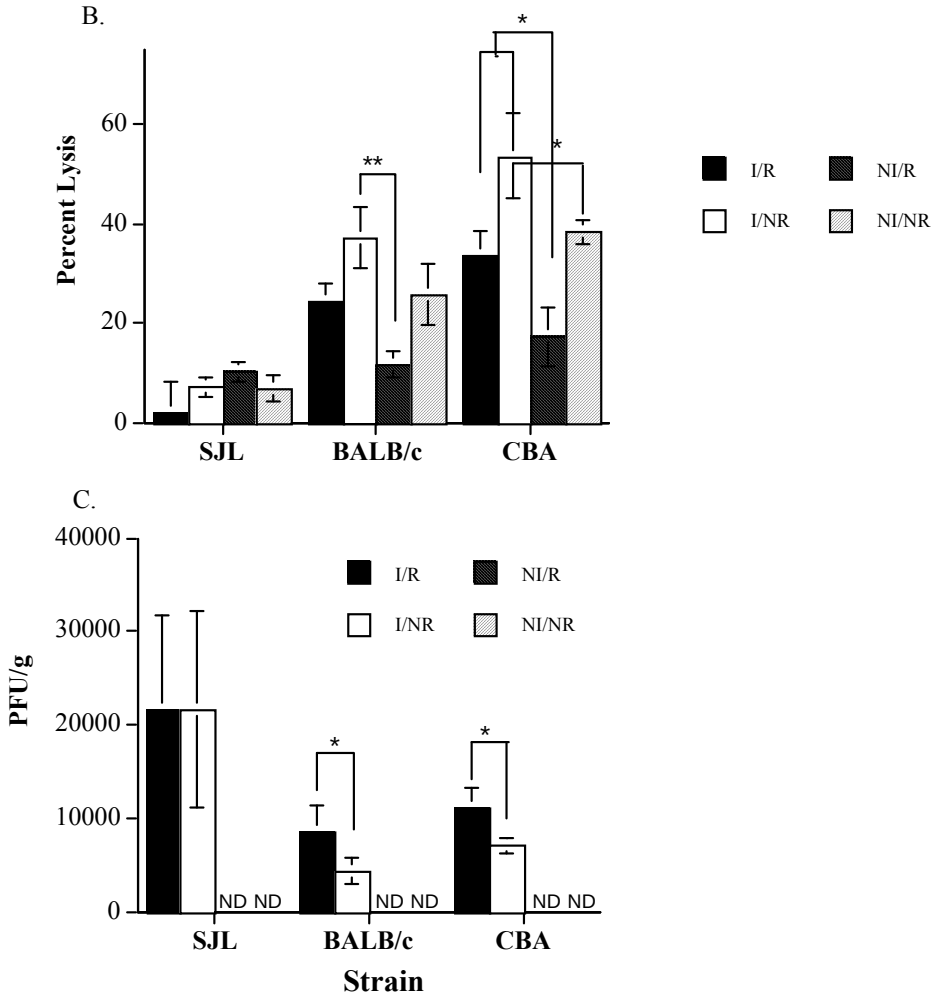


Figure 2-3, continued.

3.4 Effects of restraint stress and TMEV infection on viral load in the blood and brain.

Plaque assays were used to measure viral titers in the blood and brain. There was no virus detected in the blood of any samples (data not shown). However, infection increased viral titers in the brains of all strains tested ($p < 0.0001$). No virus was detected in any of the non-infected groups. SJL mice appeared to have more virus in their CNS than either CBA or BALB/c mice (Fig. 2-3C), but this trend failed to reach

statistical significance ($p = 0.0774$). Even though restraint stress did not influence the quantity of virus present in the brains of SJL mice, there was found to be an interaction between infection and restraint stress, such that restraint stress significantly increased the viral load in the brains of both BALB/c and CBA mice $p < 0.05$ (Fig. 2-3C). Because restraint stress increased viral load in CBA and BALB/c mice while at the same time decreased overall NK cell activity in the spleen, we were interested in whether or not there was a correlation between splenic NK cell activity and viral load in the brain. Simple regression analysis produced a negatively sloped effect, but this effect was not significant ($p = .2563$).

4. Discussion

Experimentally, chronic stress can hinder the generation of an immune response to many types of viral infections. In particular, restraint stress has been shown to reduce levels of NK cell cytotoxicity following infection with either herpes simplex virus (Bonneau et al., 1991), influenza virus (Hunzeker et al., 2004; Tseng et al., 2005) or TMEV (Welsh et al., 2004). While glucocorticoids are thought to alter the cellularity of NK cells in lymphoid organs such as the spleen, the mechanism by which restraint stress decreases NK cell cytotoxicity is thought to be directly related to the μ-opioid receptor since the administration of β-funaltrexamine, but not nor-binaltorphimine or naltrindole, has been shown to restore NK cell cytotoxicity (Tseng et al., 2005). In the current study, we found that RS decreased splenic NK cell cytotoxicity in BALB/c and CBA mouse strains, but did not affect SJL NK cell cytotoxicity. However, the latter mouse strain is

known to have an NK cell deficiency (Kaminsky et al., 1987), making detection of differences in NK cell cytotoxicity between treatment groups difficult. Moreover, it appeared that decreased splenic NK cell effector function corresponded to increased viral titers in the brains of infected mice (Fig. 2-3B-C). SJL mice had the lowest NK cell response, which tended to correspond to the highest viral titer, although this did not reach statistical significance. It is important to remember that these mice had only been infected for one day (see methods and materials). Therefore, it is likely that the large standard deviations from the means of viral titers in the CNS of SJL mice is due, in part, to viral replication and dissemination and not just sample variation**.**

To date, we have shown that chronic restraint stress results in an overall negative effect on the immune function (Campbell et al., 2001; Welsh et al., 2004; Mi et al., 2004) which is most clearly demonstrated by the finding that RS mice have increased viral titers in most organs (Mi et al., 2006a) as well as the central nervous system (Campbell et al., 2001). Recently, Sieve et al. have shown that, as a result of RS, immune impairment in the early phase of TMEV infection, coupled with increased viral replication leads to an earlier onset and exacerbated demyelinating disease as evidenced by both clinical and histological findings in both male and female SJL mice (Sieve et al., 2004). However, in CBA mice, a sex-dependent effect of stress was observed such that previously stressed female mice developed more severe demyelinating disease, whereas previously stressed males actually showed improvements during the chronic phase of TVID (Sieve et al., 2006). Since restraint-stress induces approximately 50% reduction

in NK cell activity in both male and female CBA mice (Welsh et al., 2004; and current study), NK cell activity is unlikely to be the sole factor in determining disease outcomes.

In this study we found that the non-susceptible BALB/c strain of mice have higher basal serum TNF-α levels than either the semi-susceptible CBA or susceptible SJL mice (Fig. 2-2B). This increased TNF-α level may play an important role in providing innate resistance to TMEV since TNF-α has been shown to have anti-viral properties (Welsh et al., 1995). In contrast to our previous work, we did not observe significant stress-induced elevations in CBA serum TNF-α levels (Welsh et al., 2004). Even though RS CBA mice appeared to have higher mean values of serum TNF-α levels than did their non-RS counterparts, this effect did not reach statistical significance (Fig. 2-2A). While, the previous study employed male CBA mice (Welsh et al., 2004), the current study employed female CBA mice. Most recently, in a direct comparison study of the effects of chronic stress and gender on TMEV-induced demyelination, we have found that RS had a greater effect on male CBA body mass, encephalitis and behavioral psychology measurements than female CBA mice in the acute phase of infection with TMEV (Sieve et al., 2006). Therefore, the reason for this TNF-α discrepancy between studies, in our opinion, can most likely be contributed to gender**.**

The non-specific innate immune response plays an important role in the early protection against infections (Tosi, 2005). Even though the innate immune response is able to limit infectious pathogens directly through the actions of epithelial cell barriers, neutrophils, macrophages/monocytes, eosinophils, basophils, mast cells, NK cells, defensins and complement, the innate response also effects the generation of adaptive

immunity through the stimulation of T-cells and B-cells. For instance, IL-12 secretion from macrophages and dendritic cells stimulates NK cells to secrete IFN-γ and enhances cytolytic activity. IFN-γ in turn, can activate macrophages, as well as, directly influence antiviral CD4 and CD8 responses (Whitmire et al., 2005a; 2005b). Alternatively, IL-4 secretion from mast cells can stimulate T-cells to produce anti-helmenthic properties and the generation of humoral responses. Thus, the impedance of this arm of immunity, by stress, in TMEV-infected mice would certainly alter the generation of adaptive immune responses leading to increased viral replication. However, in this study we were unable to detect any circulating IFN-γ. In light of the fact that we were able to detect differences in NK cell mediated lysis *in vitro*, future studies should be designed to investigate the effects of RS on the ability of NK cells to produce and secrete IFN-γ following *in vitro* stimulation.

In summary, this study focused on the effect of chronic restraint stress on the NK cell-mediated cytotoxic response in three mice strains of varying susceptibility to TMEV infection. Our results indicated that chronic restraint stress can decrease splenic NK cell responses while at the same time lead to increased viral titers in the brains of acutely infected CBA and BALB/c mice. In a model of MS that is dependent on viral persistence, increased viral titers in the acute phase of infection result in an earlier onset and exacerbated chronic phase of disease. While NK cells certainly play a role in limiting viral infection, they are not completely responsible for clearing TMEV from the CNS of resistant mice (Paya et al., 1989; Lin et al., 2003). As such, it becomes increasingly important to determine the effects of restraint stress on the effector

functions of adaptive immune responses including T-cell and B-cell, since they play a more important role in viral clearance, and resistance to demyelination (Oleszak et al., 2004).

III RESTRAINT STRESS MODULATES VIRUS-SPECIFIC ADAPTIVE IMMUNITY DURING ACUTE THEILER'S VIRUS INFECTION

1. Introduction

It has been estimated that multiple sclerosis (MS) affects about 350,000 people in the United States alone (Anderson, et al., 1992), around 2.5 million world wide (Compston and Coles, 2002), and that its incidence is increasing (Noseworthy et al., 2000). MS, characterized first by Charcot in the mid 1800s, is a demyelinating and neurodegenerative disorder that targets the central nervous system (CNS) and is autoimmune in nature. MS is rarely a life threatening condition (Redelings et al., 2006), but it is almost always debilitating, and causes a wide variety of symptoms including optic neuritis, muscle weakness, gait ataxia, cognitive difficulty, pain, fatigue, depression, incontinence and sexual dysfunction (Noseworthy et al., 2000).

While the cause of MS is not known, it is evident that multiple factors are likely to contribute to the pathogenesis of this disease, including, gender, genetic background, and environment (Noseworthy et al., 2000). No single known pathogen has been associated exclusively with the development of MS, but many different pathogens have been isolated from MS plaques (Fazakerley and Walker, 2003). Other evidence for the involvement of an infectious agent in MS development are epidemiological (Kurtzke et al., 2001), migrational (Gale and Martyn, 1995), and the pathological characterization of MS plaques (Lucchinetti et al., 2000).

Another environmental factor that may contribute to both the onset and exacerbation of MS is psychological stress (Ackerman et al., 2003; Mohr et al., 2004). Stress, acting through the hypothalamic-pituitary-adrenal (HPA) axis as well as the autonomic nervous system, has been shown to modulate immune responses in a bi-directional fashion with acute stress stimulating leukocyte trafficking and chronic stress being immunosuppressive (Dhabar and McEwen, 1997). Recently, Mohr and Pelletier have proposed that HPA-axis dysregulation by the following three processes could contribute to the pathogenesis in MS (Mohr and Pelletier, 2006): First, stress resolution could result in uncontrolled inflammation. Secondly, glucocorticoid resistance could lead to unregulated activation of autoimmune cells. Thirdly, stress-induced mast cell activation may result in increased blood-brain barrier permeability allowing autoimmune cell infiltration (Mohr and Pelletier, 2006).

We have recently been interested in the mechanisms by which restraint stress can alter the pathogenesis of Theiler's murine encephalomyelitis virus (TMEV) infection, a commonly used animal model of human MS (Lipton, 1975; Oleszak et al., 2004). In this model, intracranial injection of TMEV into susceptible strains of mice, its natural host, can lead to a biphasic disease consisting of a polioencephalitic phase followed by a chronic demyelinating phase that is perpetuated by T-cell and B-cell autoimmune responses (Oleszak et al., 2004). Conversely, viral inoculation of genetically-resistant strains of mice does not result in demyelination.

After infection with TMEV, susceptible mice fail to generate a well-orchestrated immune response, which allows the virus to persist, primarily in

macrophages/monocytes as well as astrocytes, for the duration of the animal's life time (Theiler, 1937; Clatch et al., 1990; Zheng et al., 2001). Therefore, viral persistence is a prerequisite for demyelination (Brahic et al., 1981; Rodriguez et al., 1996). Susceptibility to TMEV-induced demyelination is attributed to several factors (reviewed by Brahic et al., 2005) including the H-2D locus (Lipton and Melvold, 1984; Rodriguez and David, 1985), indicating a role of virus-specific $CD8^+$ T-cells in the clearance of TMEV. Findings in support of this hypothesis are: (i) deletion of $CD8^+$ cells by antibody administration exacerbates disease (Borrow et al., 1992), (ii) β-2 microglobulin deficient mice (Begolka et al., 2001; Fiette et al., 1993; Miller et al., 1995; Pullen et al., 1993) as well as (iii), perforin-deficient (Palma et al., 2001; Rossi et al., 1998; Murray et al., 1998) mice generated on a Theiler's virus-induced demyelination resistant background, are rendered susceptible. The generation of TMEV specific antibodies has also been shown to contribute to viral clearance (Welsh et al., 1989; Kang et al., 2005).

The effector function of the Th cell is to facilitate the production of adaptive immune responses. However, while Th1 type immunity is essential for early TMEV clearance by the generation of both $CD8^+$ T-cell and B-cell responses; in the later phase of TMEV Th1 mediated immunity is damaging to the CNS. Nevertheless, pro-inflammatory responses early following infection are beneficial. In fact, in CBA mice, deletion of $CD4^+$ T-cells during the time of infection was shown to cause mortality (Welsh et al., 1987). Similarly, in $CD4^+$ T-cell deficient SJL mice infected with the DA strain of TMEV the disease was exacerbated (Lin et al., 2004).

Restraint stress (RS) has been shown to be immunosuppressive in several viral infections including herpes simplex virus (Bonneau et al., 1991) and influenza virus (Feng et al., 1991). We have demonstrated that RS can dramatically influence the disease course of TMEV. Particularly, SJL mice that received 4 weeks of 8 hour nightly RS develop an exacerbated disease course (Sieve et al. 2004) with similarities to that generated following depletion of $CD8^+$ T-cells via monoclonal antibody administration (Borrow et al., 1992). Moreover, infected CBA mice that received nightly RS of 12 hours produced a survival curve (Campbell et al., 2001) that was almost identical of that generated by the depletion of $CD4^+$ T-cells (Welsh et al., 1987). Because of these similarities, we hypothesized that restraint stress during acute infection with the BeAn strain of TMEV alters the generation of specific immune responses. Therefore, in this particular study we set out to determine the effects of restraint stress on the generation of virus specific $CD4^+$ T-cell, $CD8^+$ T-cell and B-cell effector functions.

2. Methods and materials

2.1 Mice

Three to four week old female SJL mice were purchased from Harlan (Indianapolis, IL). Mice were assigned to either infected restrained (I/R), infected non-restrained (I/NR), non-infected restrained (NI/R) or non-infected non-restrained (NI/NR) groups (Mi et al., 2004; 2006a). For some experiments, mice were assigned to infected/ PBS treated (I/PBS), infected/dexamethasone treated (I/DEX), infected/restrained/PEG400 treated (I/R/PEG) or infected/restrained/RU486 treated

(I/R/RU486) groups. Each mouse was identifiable by unique cage number, and markings on made on the tale. For each individual experiment mice were housed two animals per cage per group. All mice were given one week to acclimate to their housing environment (light dark cycle 0500- 1700 hr) prior to the onset of the experiment. All mice were kept on a diet of mouse chow containing 9% fat and 20.5% protein. Food and water was provided *ad libitum*. All experiments were approved by the animal use and care committee at Texas A&M University.

2.2 Virus, Viral purification and inactivation, and viral peptides

The BeAn 8683 strain of Theiler's virus (kindly provided by Dr. H. L. Lipton, Department of Microbiology-Immunology, University of Illinois at Chicago, IL) was initially propagated in lung tumor (L2) cells (Welsh et al., 1987). Virus was semi-purified by ultracentrifugation according to (Rueckert and Pallansch, 1981) by pelletting through 30% sucrose cushion at 80,000 x g for 3 hours in a Optima L-80 XP ultracentrifuge (Beckman Coulter) using an SW-28 rotor. Pellets were resuspended in 1M sodium phosphate buffer (pH 7.4). UV-inactivated virus was generated by exposing previously pelleted virus to UV-light (1330 μW/cm3 at 13 cm distance) for 1.5 hours. The virus was determined to be inactivated by plaque assay.

The immunodominant $CD4^+$ T-cell peptide QEAFSHIRIPLPH corresponding to TMEV $VP2_{74-86}$ was used to determine $CD4^+$ T-cell specific responses (Gerety et al., 1991 and 1994). Additionally, the immunodominant $CD8^+$ T-cell peptide FNFTAPFI corresponding to $VP3_{159-166}$ was used to determine $CD8^+$ T-cell specific responses to

TMEV (Kang et al., 2002). The non-specific peptide sequence RLNRITKDSYPNS was used as a control peptide to determine non-specific immune responses. All peptides were purchased from Sigma at ≥ 95% purity.

2.3 Infection and restraint stress

Following anesthesia with isoflourane (Vedco, MO, USA), mice were either injected with 5.0 x 10^5 plaque forming units (PFU) of BeAn strain of TMEV in 20 μl of DMEM media or sham-infected with DMEM into the right mid-parietal cortex at a depth of approximately 1.5mm (Welsh et al., 1987; Campbell et al., 2001; Mi et al., 2004; Sieve et al., 2004). Restraint stress was carried out as described previously (Sieve et al., 2004). Restraint sessions began the night prior to infection and continued each night until day 8 p.i. All mice were allowed two hours to recover prior to injection. Mice were weighed daily.

2.4 Physiological indices of infection and stress

Mice were monitored and scored daily for signs of sickness. Starting 2 days prior to infection mice received clinical scores based on degree of ruffling, hunching, weakness, wobbly gait and righting reflex, where 0 = healthy and 4 = moribund for each measure (Sieve et al., 2004). Also, body mass of all animals was recorded daily, and abdominal body temperatures were measured daily using an infraRed + Type K thermometer (Waltham, MA) starting one day prior to infection and lasting for the

duration of the experiment. All temperature readings were taken in triplicate and at 1200 hr each day.

2.5 Activity monitoring

Mice were monitored for behavioral differences at days –1, 3 and 5 post infection (p.i.) as described previously (Johnson et al., 2006). Briefly, each mouse was placed in the open field monitoring area between the hours of 0700 hr and 1000 hr. All horizontal and vertical movements were recorded each minute for a total of 20 minutes per session.

2.6 Dexamethasone and RU486

For some experiments, mice were treated nightly with dexamethasone (DEX) (Sigma Chemical, St. Louis, MO) at a dose of 1.0 mg/kg or with vehicle control (PBS) by intraperitoneal (i.p.) injection given 1 hour prior to restraint. RU486 (Sigma Chemical, St. Louis, MO) treatment was administered according to Tseng et al., (2005) such that mice were given nightly subcutaneous (s.c.) injections of RU486 (25mg/kg) or vehicle control (PEG400). Both DEX and RU486 treatments were administered 1 hour prior to restraint, began the night prior to infection, and lasted until day 8 p.i. All injection volumes were administered in a total volume of 100 µl.

2.7 Tissue isolation

At the termination of each experiment (Day 8 or 16 p.i.) all mice were injected with a lethal dose of Beuthanasia special 150mg/kg (Schering-Plough Animal Health)

(Welsh et al., 2004). Blood was collected via cardiac puncture, and then mice were perfused through the left ventricle with cold Hanks balanced salts solution containing heparin (10U/mL) buffered at pH 7.2. Serum was collected as described (Welsh et al., 2004). After perfusion, spleens, brains and spinal cords were aseptically removed. Single cell suspensions were prepared as described previously (Welsh et al., 2004). CNS infiltrating lymphocytes (CNS-ILs) were prepared from the CNS tissue using nylon mesh and incubating the tissue with RPMI 1640 (supplemented with 2% FBS) containing 250μg/ml of Collagenase type IV (Worthington Inc., Lakewood, NJ) for 45 minutes at 37°C and 5% CO_2 (Kang et al., 2002a). Following incubation, the lymphocytes were isolated by percoll gradient centrifugation (Irani and Griffin, 1991), then re-suspended in complete RPMI-1640 containing L-glutamine (1.0%), penicillin and streptomycin (1.0%), and 10% FBS.

2.8 Plaque assay

UV-inactivated virus was determined to be inactivated by plaque assay as described previously (Mi et al., 2006b).

2.9 Preparation of feeder cells

Spleens were aseptically removed from aged-matched NI/NR mice and single cell suspensions were prepared. Feeder cells were irradiated with 3000 rads (Co^{60} source, College of Veterinary Medicine, Texas A&M University).

2.10 ELISPOT assays

The effects of restraint stress on T-cell effector function to TMEV were assessed, in part, by the ability of CNS-ILs to generate IFN-γ in response to either the immunodominant $CD4^+$ or $CD8^+$ T-cell specific peptide ($VP2_{74-86}$ or $VP3_{159-166,}$ respectively). In this assay, sterile flat-bottomed 96-well filtration plates (MAIPS4510) containing PVDF membranes (Millipore, Corp, Bedford, MA) were coated with 1.0 μg (in 100μl sterile PBS) of anti-mouse IFN-γ (AN-18) (eBioscience) capture antibody (eBioscience) overnight at 4°C. The plates were blocked with 200μl complete RPMI-1640 (supplemented with 10% FBS) for 2 hours at room temperature. Then 2.0×10^4 CNS-ILs were mixed with 1.0×10^6 irradiated feeders, in 150 μl of complete RPMI-1640 with a final concentration of 2.0 μM peptide ($CD4^+$, $CD8^+$ or Non-specific) and then added to the plate. Following an incubation at 37° C and 5.0% CO_2 for 24 hours the plates were washed with PBS containing 0.05% Tween-20 (5X) and rinsed once with water purified by reverse osmosis (RO) H_2O. 100 μl assay diluent (PBS with 10% FBS) containing 0.1μg of the biotin labeled anti-IFN-γ detection antibody (R4-6A2) (eBioscience) was added to each well, and the plates were incubated at room temperature for 2 hours. After the incubation the plates were washed 6X with PBS containing Tween-20 (0.05%). Then, 100μl of avidin-HRP (horseradish peroxidase) (eBioscience) diluted 1/1000 in assay diluent was added to each well and the plates were incubated for 30 minutes at room temperature. After washing 6X as described above, spots were developed using 100 μl of 3-amino-9-ethyl-carbazole (AEC) substrate solution (1.0mg AEC, 1.0ml dimethylformamide, 14ml 0.1M citrate-phosphate buffer

pH 5.0, and 10.0 µl and H_2O_2). After development, the plates were rinsed 3X with 200µl of RO H_2O, and read with an ELISPOT plate reader (AID EliSpot Reader System, Straberg, Germany). The effects of RS on splenic T-cell effector function were determined using the methods described above. However, for these assays, 1.0 x 10^6 isolated spleen cells were used in the absence of feeders. All samples were run in duplicate. Background was determined as the generation of spots to the non-specific peptide and was subtracted from $CD8^+$ and $CD4^+$ T-cell virus-specific peptide responses.

Virus-specific antibody secreting cells (ASCs) were determined using a modification of the above assay. Briefly, 100 µl of previously pelleted virus (10 ug/ml) in sterile PBS was used to coat each well overnight. After blocking, either 2.0 x 10^4 CNS-IL or 1.0 x 10^6 splenocytes were added to each well, and the plates incubated for 24 hours as described above. After washing, biotinylated anti-mouse Ig (Jackson) was added as described (Pachner et al., 2007) and incubated for 2 hours at room temperature. Plates were washed again, then incubated with avidin-HRP for 30 minutes at room temperature. The plates were then developed with AEC substrate and spots enumerated as described above.

2.11 Flow cytometry

Flow cytometry was used to test the effects of RS on splenic T-cell percentages, at days 8 and 16 p.i. Briefly, 1.0 x 10^6 splenocytes were blocked with 1.0 µg of anti-mouse CD16/CD32 (93; eBioscience) for 10 minutes at 4°C then labeled with PE conjugated anti-CD3e (145-2C11; eBioscience), and FITC conjugated anti-CD4 (RM4-

5; eBioscience) or FITC conjugated anti-CD8a (53-6.7; eBioscience) for 20 minutes at 4°C. Cells were then washed 3X with staining buffer (eBioscience) then resuspended with 300µl EM grade paraformaldehyde (Electron Microscopy Science; Ft Washington, PA). T-cell percentages were then determined using a FACScaliber (Becton Dickson) and MoFlow software.

2.12 Cytokine secretion

At day 8 p.i. 5 x 10^4 CNS-IL and 1.0 x 10^6 irradiated splenocytes or 1.0 x 10^6 splenocytes were cultured with complete RPMI 1640, and stimulated with either plate bound anti-CD3 (10 µg/ml) and anti-CD28 (2.0 µg/ml), purified UV-irradiated BeAn, or control media for 72 hours at 5.0% CO_2 and 37°C. The supernatants were then collected and concentrations of Th1, Th2 and Th17 cytokines measured using Bio-plex kits (Th1/Th2) or ELISA (Th17) according to the manufacturers' instructions.

For some experiments, the effects of RS or dexamethasone treatment on virus specific IFN-γ secretion was measured by ELISA (eBioscience) after stimulating 1.0 x 10^6 splenocytes (isolated at day 8 p.i.) for 24 hours with 2µM of $VP2_{74-86}$, $VP3_{159-166,}$ or non-sense (NS) peptide with or without DEX in complete RPMI 1640 at 5.0% CO_2 and 37°C as indicated in the text.

2.13 Effect of dexamethasone on T-cell proliferation

To determine the effects of DEX on T-cell proliferation, 1.0 x 10^6 splenocytes isolated from I/NR mice at day 8 p.i. were stimulated with media, plate bound anti-CD3

(10μg/ml) and anti-CD28 (2.0μg/ml) antibodies or peptides in the presence of DEX. After 24 hours of stimulation at 37° C and 5.0% CO_2, 20 μl MTT (Sigma) at a concentration of 5mg/ml dissolved in PBS was added to each well and the plate was incubated for 4 hours at 37° C and 5.0% CO_2. Next, 50 MTT lysis buffer (50% dimethylformamide, 20% SDS) was added to each well and the plate was incubated overnight at 37° C with 5.0% CO_2. Absorbance was determined at 570 nm in a FLUOstar Optima spectrophotometer (BMG Inc., Offenburg, Germany).

2.14 Serum cytokine profile

At day 8 p.i., the effects of RS on serum cytokine concentrations were determined using a Bio-Plex 23 plex profiling kit according to the manufacturer's instructions (Bio-Rad, Inc; Hercules, CA). This kit gave the concentrations of 23 serum cytokines including IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, Eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α.

2.15 Quantitative RT-PCR and western blots for T-bet and GATA-3

Total RNA was isolated from 1.0 x 10^7 spleen cells by QIAshredders (Qiagen), and purified by column and DNase digestion using a Qiagen RNeasy mini kit according to the manufacturer's instructions (Qiagen). cDNA was generated as described previously, albeit using random primers (Promega) (Mi et al., 2006a). Real-time PCR was used to test for IL-4, IL-5, IFN-γ, T-bet, and GATA-3, expression and normalized to

β-actin using methods described previously (Mi et al., 2006a). All primers were purchased from Integrated DNA Technologies (Coralville, IA) and sequences can be seen in Table 3-1.

To determine the effects of RS on T-bet and GATA-3 protein levels. 1.0×10^6 splenocytes were cultured with plate bound anti-CD3 (10 μg/ml) and anti-CD28 (2 μg/ml) antibodies, or 2 μM peptides $VP2_{74-86}$, $VP3_{159-166}$, or NS in 150 μl of complete RPMI-1640 for 5 or 24 hours at 37°C and 5% CO_2. Nuclear protein was extracted from stimulated splenocytes using NU-PER kits (Pierce), quantified by BCA protein assay (Pierce), separated on 10% polyacrylamide gels, transferred to nitrocellulose, blocked with 5% NFDM, probed with anti-mouse T-bet (4B10; Santa Cruz) or GATA-3 (HG3-31; Santa Cruz) followed by ImmunoPure anti-mouse IgG-HRP (Pierce) and developed with SuperSignal west pico chemiluminescent substrate (Pierce). Blots were then stripped and re-probed with anti-mouse β-actin (a kind gift from Dr. Weston Porter; Texas A&M University). Film (CL-XPosure; Pierce) was developed using an autoprocessor, and densitometry analysis was performed with using a Bio-Rad Analyzer gel 2K system and quantity-one software.

Table 3-1 RT-PCR pimer sequences

Target	Direction	Sequence	Reference
T-bet	Forward	5' - CAA CAA CCC CTT TGC CCA AAG - 3'	Yamamoto et al., 2005
	Reverse	5' - TCC CCC AAG CAG TTG ACA GT - 3'	
IFN-γ	Forward	5' - CAG CAA CAG CAA GGC GAA A- 3'	Mi et al., 2006
	Reverse	5' - CTG GAC CTG TGG GTT GTT GAC - 3'	
GATA-3	Forward	5' AGA ACC GGC CCC TTA TCA -3'	Yamamoto et al., 2005
	Reverse	5' AGT TCG CGC AGG ATG TCC - 3'	
IL-4	Forward	5' - ACA GGA GAA GGG ACG CCA T - 3'	Wei et al., 2005
	Reverse	5' - GAA GCC CTA CAG ACG AGC TCA - 3'	
IL-5	Forward	5' - CCG AGC TCT GTT GAC AAG CA - 3'	Wei et al., 2005
	Reverse	5' - CAG TAT GTC TAG CCC CTG AAA GAT TT - 3	
β-Actin	Forward	5' - GCA ACG AGC GGT TCC G - 3'	Mi et al., 2006
	Reverse	5' - CCC AAG AAG GAA GGC - 3'	

2.16 Antibody levels to TMEV

Antibody levels to TMEV were determined by ELISA. Here 96-well flat-bottomed high-binding polystyrene plates (Corning Inc, Corning, NY) were initially coated with purified virus. Virus was diluted in carbonate buffer (pH 9.6), then added to the plates such that the concentration was approximately 5.0 x 10^6 p.f.u./well in 100µl. The plates were then incubated overnight at 4°C, followed by, washing with phosphate buffered saline (pH 7.2) containing Tween-20 (0.05%) and rinsing with RO-H_2O. The plates were then blocked by adding 300µl of Superblock buffer (Pierce) containing Tween-20 (0.05%) 3X, according to the manufacturer's instructions. Next, serum was diluted in assay buffer as described previously (Sieve et al., 2004). After incubating at 37°C for 1.5 hours, the plates were again washed and rinsed. Either the peroxidase-conjugated polyvalent goat anti-mouse antibody (A-0412), goat anti-mouse IgG (A-5278) or goat anti-mouse IgM (A-8786) obtained from Sigma and diluted 1/500 in assay buffer was added to each well or the plate was incubated at 37°C for 1 hour. Finally, after washing and rinsing as described, the reaction was developed using 200 µl OPD substrate (Sigma) in citrate buffer (pH 5.0) for 30 minutes and stopped with 50 µl of 2.5 M sulfuric acid. OD was determined at 490 nm using a FLUOstar Optima spectrophotometer (BMG Inc., Offenburg, Germany).

2.17 Statistical analyses

Data are presented as mean ± SEM. Data were analyzed using analysis of variance (ANOVA) and students *t*-tests. Significance of correlations was determined by simple regression analysis. In all cases significance was set at $p \leq 0.05$.

3. Results

3.1 Physiological and behavioral measurements of infection and stress

3.1.1 Theiler's virus infection and restraint stress produce distinct physiological symptoms

Prior to restraint and infection, all mice were scored, weighed, and had their abdominal temperature recorded. It was determined that both stress and infection produced changes in these physiological parameters. Specifically, mice in the RS groups had increased average clinical scores, beginning immediately following the first session of RS (Fig. 3-1A). These effects were mostly attributable to increased ruffling and grooming scores, and did not reflect sickness-induced by TMEV infection as evidenced by the fact that infection had no effect on clinical manifestations (Fig. 3-1A).

Alternatively, both RS, and infection were found to cause an overall decrease in body weight (ps < 0.001; Fig. 3-1B). Moreover, as illustrated in Figure 3-1B, these effects were dependent on time. Specifically, the effects of infection on change in body mass percentage were transient, reaching maximum at day 1 p.i. and resolving thereafter. However, RS caused continual weight loss, which was sustained throughout the course

of the experiment (Fig 3-1B). RS exacerbated the infection induced weight loss, which was illustrated by the significant day x RS x infection interaction, $p < 0.01$.

Daily abdominal temperature readings showed that Theiler's virus infection caused hypothermia, $p < 0.01$ (Fig. 3-1C). In contrast, RS showed a very strong trend towards inducing hyperthermia, but this trend failed to reach statistical significance ($p = 0.0542$; Fig. 3-1C). No other differences were found.

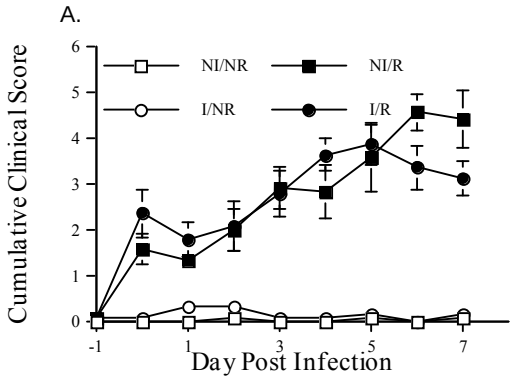


Figure 3-1. The effects of TMEV infection and RS on physiological indices of homeostasis. Groups are illustrated as: infected/non-restrained (I/NR; open circles); infected/restrained (I/R; closed circles) non-infected/non-restrained (NI/NR; open squares); non-infected/restrained (NI/R; closed squares). A. Restraint stress alone significantly increased clinical scores. B. Both TMEV infection and RS altered body mass. C. Infection and RS altered abdominal temperatures throughout the course of infection. Results are combined means of 3 independent experiments and are expressed as means ± S.E.Ms.

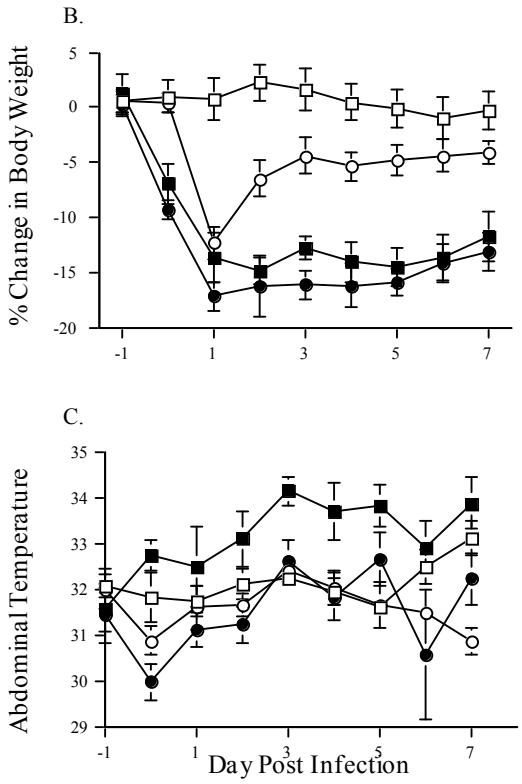


Figure 3-1, continued.

3.1.2 RS alone, but not Theiler's virus infection alone, alters vertical exploratory behavior

Behavioral data were generated as described previously (Johnson et al., 2006). No differences in baseline activity measurements existed between mouse groups (Figs. 3-2A, B, G and 3-3A, B, G). However, by day 3 post infection (p.i.), mice in the RS groups showed reduced vertical time as well as reduced numbers of vertical movements, all $ps < 0.05$ (Figs. 3-2D and 3D). Although infection with TMEV alone did not influence exploratory behavior, there was a significant interaction between infection and RS at day 3 p.i. when mice in the I/R group had decreased vertical activity (Fig. 3-2C), vertical time (Fig. 3-2D), number of movements (Fig. 3-3C) and number of vertical movements (Fig. 3-3D) when compared to mice in all other groups, ($ps < 0.05$). By day 5 p.i., the effects of RS became more evident, as RS was found to decrease the vertical activity (Fig. 3-2E-G), vertical time (Fig. 3-2F-H), the total number of movements (Fig. 3E-G), and the number of vertical movements (Fig 3-3F-H), all $ps < 0.01$. Conversely, no effects of infection were seen at this time point.

Of all measurements taken, change in body weight most adequately reflected influences of both stress and infection. Therefore, this measurement was used to indicate these conditions in repeat experiments.

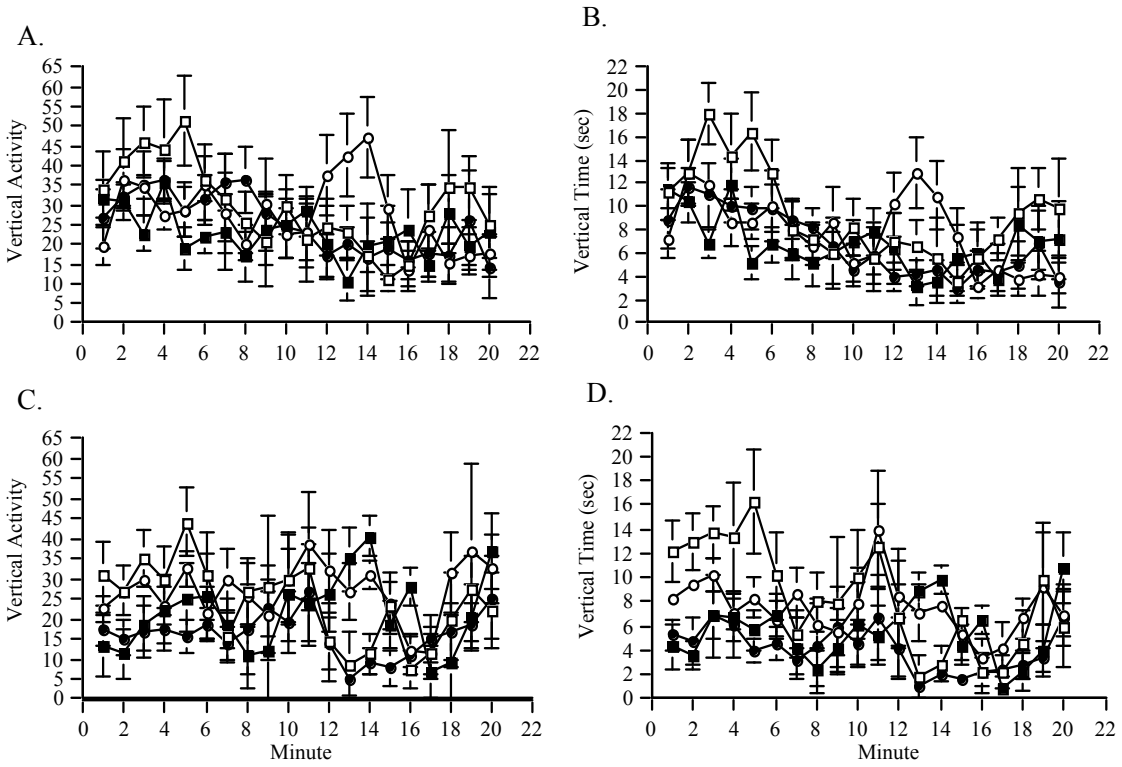


Figure 3-2. The effects of RS and TMEV infection on vertical activity. Activity was recorded for 20 min during the hours of 0700 to 1000 using a digipro machine on days –1, 3, and 5 p.i. Groups are: infected/non-restrained (I/NR; open circles); infected/restrained (I/R; closed circles) non-infected/non-restrained (NI/NR; open squares); non-infected/restrained (NI/R; closed squares). There were no differences in the amount of vertical activity or vertical time between groups at baseline (day -1p.i.) (A and B). At day 3 p.i., however, RS reduced the vertical time (D), and there were interactions between minute and infection and stress (C and D). At day 5 p.i. stress mice were disinclined to demonstrate vertical activity or vertical time (E and F). The effect of day on both vertical activity and time are represented by graphing the averages of the 20 min session and are summarized in G and H. Results are means ± S.E.Ms.

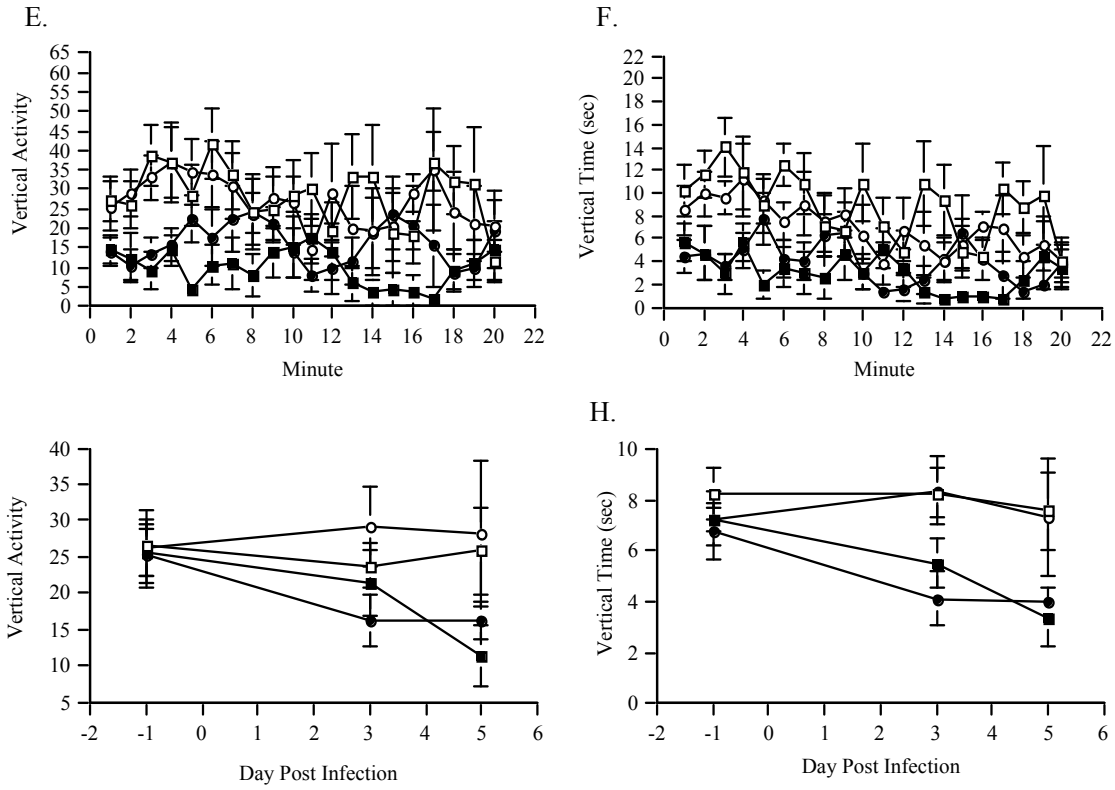


Figure 3-2, continued.

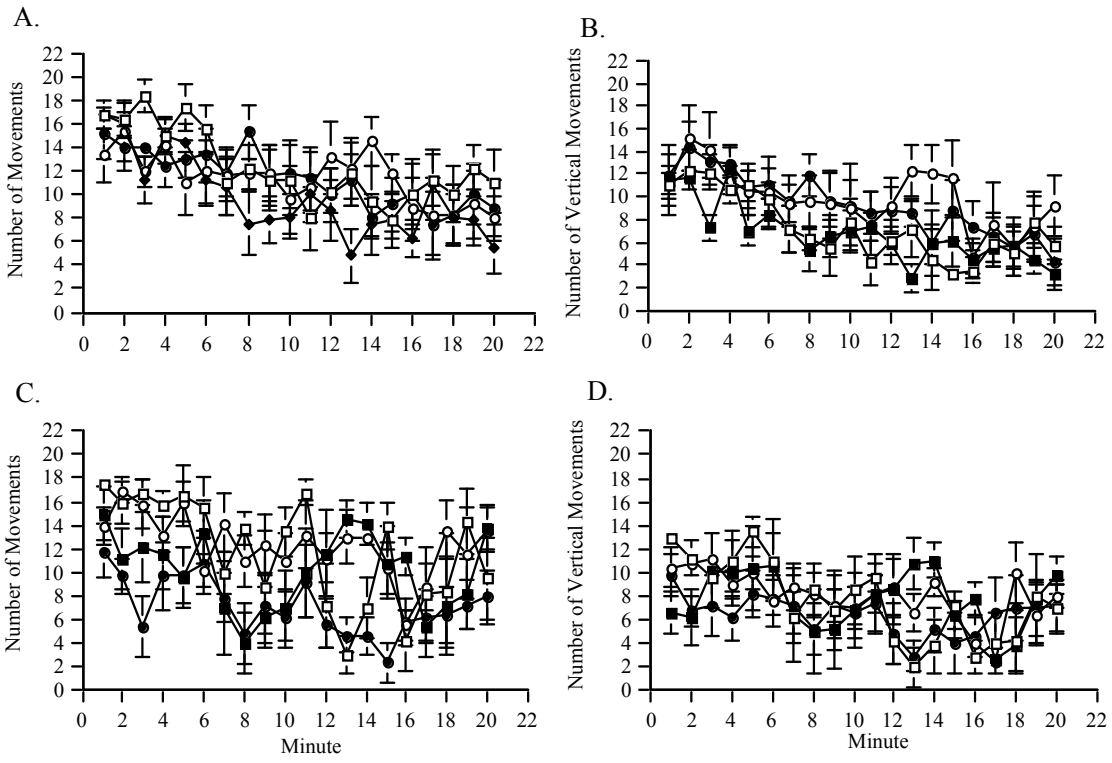


Figure 3-3: The effects of RS and TMEV infection on movement. The activity of all mice was recorded for twenty minutes during the hours of 0700 to 1000 using a digipro machine on days –1, 3, and 5 p.i. Groups are illustrated as: infected/non-restrained (I/NR; open circles); infected/restrained (I/R; closed circles) non-infected/non-restrained (NI/NR; open squares); non-infected/restrained (NI/R; closed squares). There were no differences in either total movement or vertical movement between groups at baseline (day –1p.i.) (A and B). At day 3p.i. RS reduced the total number of movements (C), but had no effect on vertical movements (D). At this time point interactions existed between stress and minute as well as stress, infection and minute for the number of movements (C). By day 5p.i. RS resulted in decreases in both the total number of movements (E) as well as the number of vertical movements (F). Averages of the 20 sessions for both number of movements (G) and number of vertical movements (H) are displayed for simplicity. Results are means ± S.E.Ms.

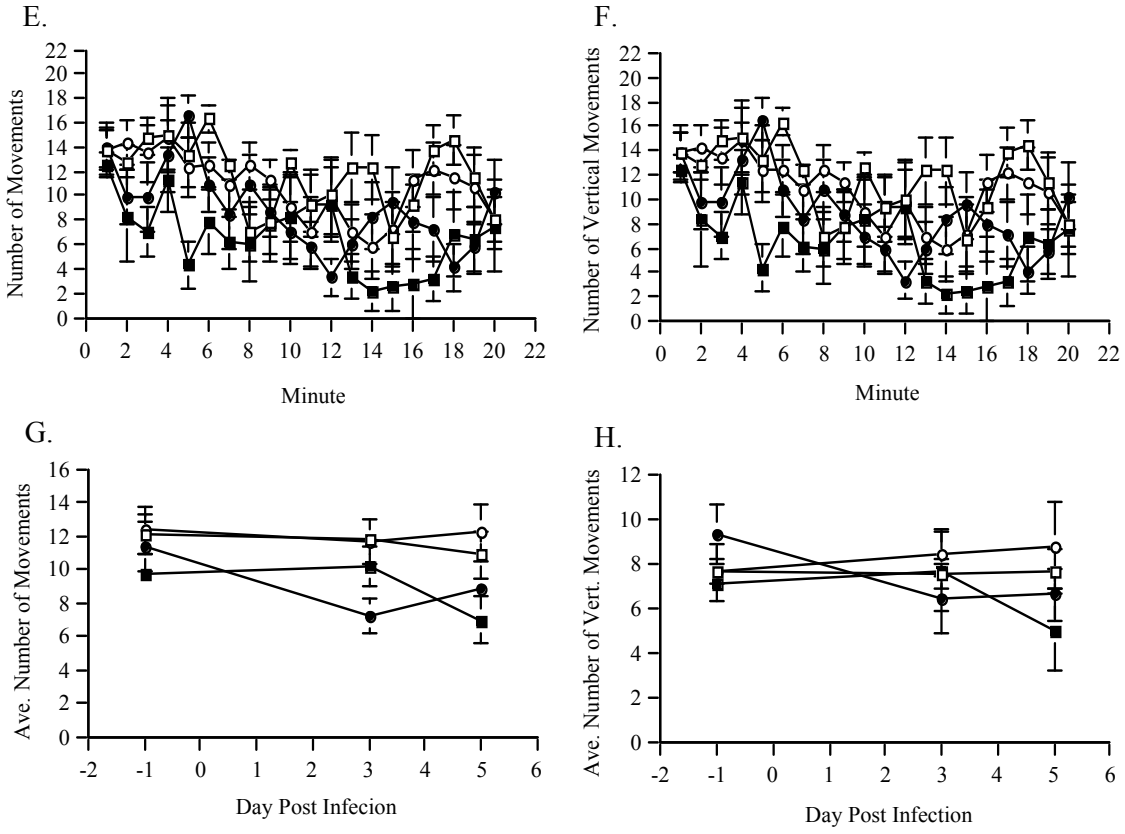


Figure 3-3, continued.

3.1 Effects of stress on the immunologic responses to infection

3.2.1 Restraint stress has a profound influence on T-cell responses in the CNS and spleens at days 8 p.i.

Cellular immune responses, particularly $CD8^+$ T-cell responses, are imperative for the clearance of TMEV from the CNS and, thus, essential for preventing the development of immune-mediated demyelination in this model of human MS. The benefit of IFN-γ in the clearance of TMEV is illustrated by the fact that IFN-$\gamma^{-/-}$ mice on

a normally resistant background succumb to viral persistence and eventually demyelination (Rodriguez et al., 2003). In order to determine the effects of RS on IFN-γ producing T-cells in both the CNS and the periphery, we utilized known immunodominant $CD4^+$ ($VP2_{74-86}$), and $CD8^+$ ($VP3_{159-166}$) T-cell epitopes to stimulate these cells in the context of an ELISPOT assay. Within the CNS, very few cells responded to the $CD4^+$ immunodominant peptide ($VP2_{74-86}$), confirming the results of others that suggest that almost no capsid specific Th1 cells are present in the CNS of TMEV infected SJL mice at this time point (Fig. 3-4A; Mohindru et al., 2006). In sharp contrast, infection caused an influx of CNS-IL that were specific for the $CD8^+$ immunodominant epitope ($VP3_{159-166}$) ($p < 0.001$) at day 8 p.i. (Fig.3-4B). Within infected animals, RS decreased the number of $VP3_{159-166}$ reactive $CD8^+$ cells by approximately 58% ($p < 0.05$). Additionally, at this same time point, infection increased the number of both $VP2_{74-86}$ ($CD4^+$) and $VP3_{159-166}$ ($CD8^+$) specific IFN-γ producing cells in the spleens of infected mice ($ps < 0.01$) (Fig 3-4C-D). While $CD4^+$ T-cell responses were different between the periphery and the CNS, it is worth noting that the $CD8^+$ T-cell responses were very similar and in fact significantly correlative ($r = 0.6481$; $p < 0.01$; see Appendix A for correlation). Importantly, restraint stress decreased both of these responses in the spleens of infected mice by approximately 50 and 60% respectively ($ps < 0.01$) (Fig. 3-4B&D). Taken together these data indicate that RS drastically reduces the number of virus-specific IFN-γ producing $CD4^+$ and $CD8^+$ T-cells at a time when these T-cell responses normally peak.

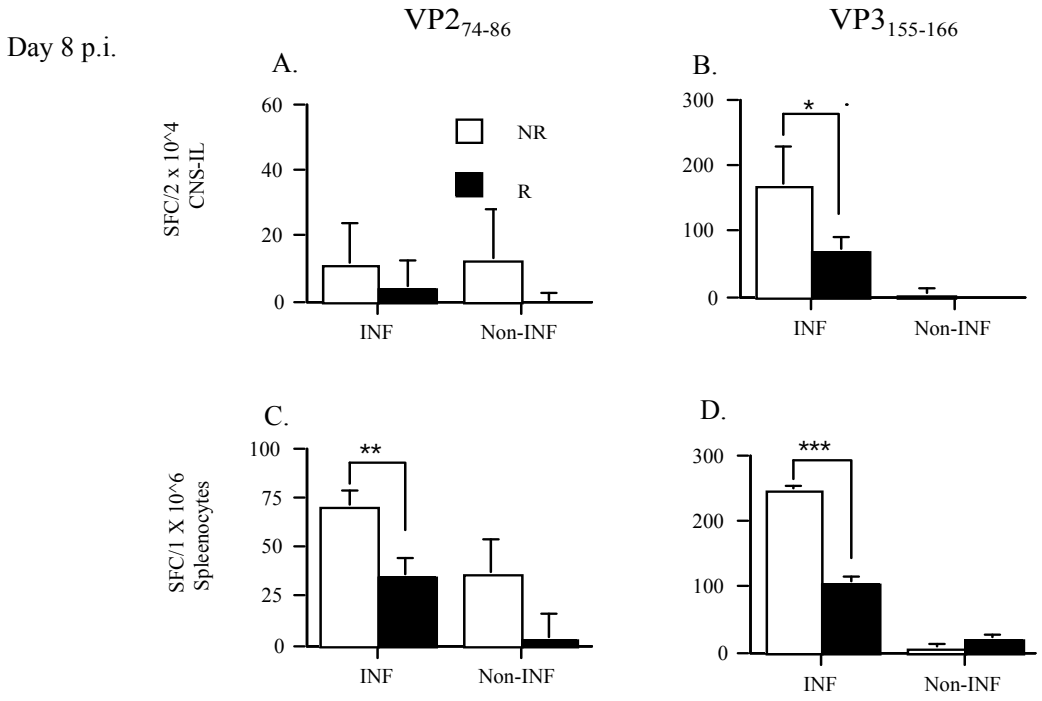


Figure 3-4. Restraint stress alters the numbers of virus specific IFN-γ producing cells in the CNS and spleens of SJL mice at days 8 and 16 p.i. Mice were assigned to either infected/non-restrained (I/NR), infected/restrained (I/R), non-infected/non-restrained (NI/NR) or non-infected/restrained (NI/R) groups. At day -1 p.i. mice in the restrained groups were subjected to 8 h nightly restraint sessions which lasted until day 8 p.i. At day 8 or 16 p.i. isolated lymphocytes were stimulated with either CD4 ($VP2_{74-86}$) or CD8 ($VP3_{159-166}$) restrictive or non-specific (NS) peptide in the context of an IFN-γ ELISPOT assay. A. IFN-γ producing cells specific for $VP2_{74-86}$ in the CNS at day 8 p.i. B. VP3 specific IFN-γ producing cells in the CNS at day 8 p.i. C. $VP2_{74-86}$ specific IFN-γ producing cells from spleens. D. $VP3_{159-166}$ reactive cells from spleens at day 8 p.i. E. $VP2_{74-86}$ specific IFN-γ producing cells from the CNS of mice at day 16 p.i. F. $VP3_{159-166}$ specific IFN-γ producing cells isolated from the CNS spleens (H) at day 16 p.i. G. $VP2_{74-86}$ specific IFN-γ producing cells in their spleens at day 16 p.i. All samples were run in duplicate. Background spots were determined for each sample using a NS peptide as a stimulus and were subtracted from results obtained from stimulation with viral peptides. Results are combined means ± SEM of 2-3 separate experiments, and represent 5-7 mice per group. Student's t-test were used to test significance. * $P < .05$, ** $P < .01$, *** $P < .001$.

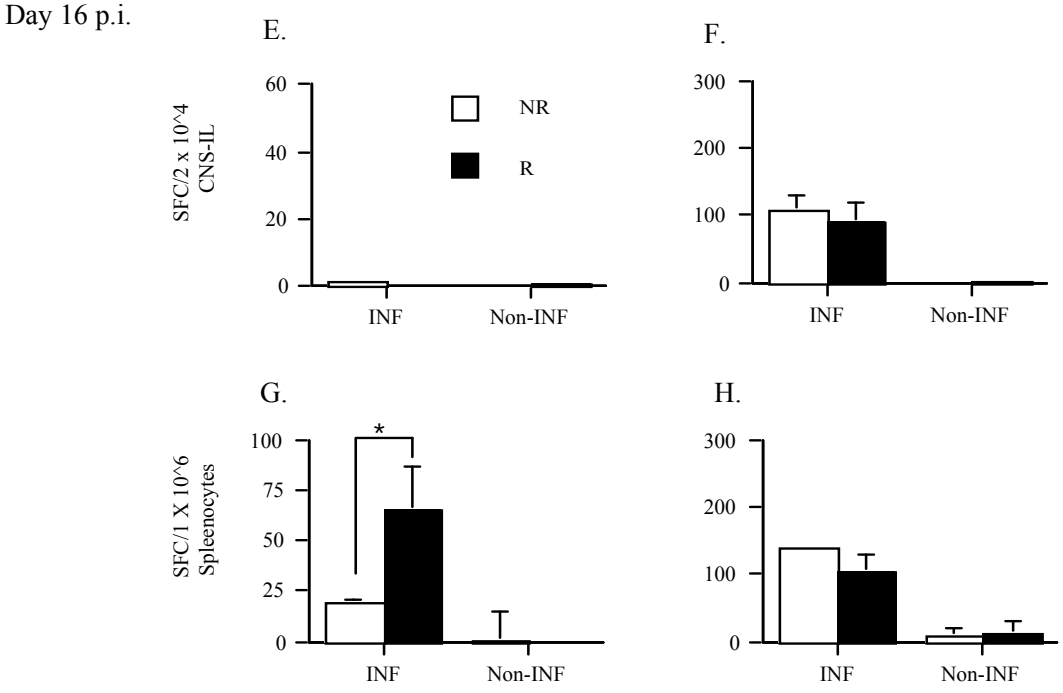


Figure 3-4, continued.

3.1.2 Recovery from restraint stress is evident by day 16 p.i.

Because overall activity, as well as body weight are rapidly returned to levels comparable to those of non-stressed mice following the secession of RS, we questioned if the immune response would be as resilient, or if the observed suppression would be sustained. Therefore, in repeat experiments, we subjected mice to 9 sessions of RS as before. At day 8 p.i. stress sessions ceased and mice were given until day 16 to recover, at which time the number of IFN- γ producing cells were measured as indicated above. Again, stimulation of CNS-IL with the $CD4^+$ immunodominant peptide, $VP2_{74\text{-}86}$, did not stimulate the production of IFN-γ indicating a lack of virus specific Th1 response in the CNS of these mice at day 16 p.i. (Fig. 3-4E). As occurred at day 8 p.i., stimulation

of CNS-IL with $VP3_{159-166}$ demonstrated high numbers of IFN-γ producing $CD8^+$ cells only in infected mice (Fig. 3-4F) at day 16 ($p < 0.001$). However, when compared to day 8 p.i. this response was lower in I/NR animals (Fig. 3-4B vs. 3-4F). Unlike at day 8, the effects of RS on $VP3_{159-166}$ specific cells from the CNS were nearly identical between non-stressed (I/NR) and stressed (I/R) groups (Fig. 3-4F). In the spleen, infected mice had increased numbers of $VP2_{74-86}$ specific IFN-γ producing $CD4^+$ cells ($p < 0.01$) at day 16 p.i. (Fig. 3-4G). Surprisingly however, the effect of stress was now reversed to almost the exact opposite of what occurred at day 8 p.i. such that non-stressed mice (I/NR) had significantly fewer $VP2_{74-86}$ reactive $CD4^+$ cells compared to stressed mice (I/R) ($p < 0.05$; Fig. 3-4C vs. 3-4G). Similar to the CNS, the numbers of $VP3_{159-166}$ specific IFN-γ producing CD8 cells in the spleens were significantly increased by infection ($p < 0.0001$) and nearly identical between non-stressed and stressed groups (Fig. 3-4H). On day 8 p.i. this response was highly correlative between the spleen and the CNS at day 16 p.i. ($r = 0.7582$; $p < 0.001$; Appendix A).

3.2.3 Restraint stress alters virus-specific antibody responses at days 8 and 16 p.i.

The effect of restraint stress on the humoral immune response to TMEV was determined using ELISA's and ELISPOT assays at days 8 and 16 p.i. Virus-specific serum antibody levels were found to be significantly increased in infected mice at day 8 p.i. when compared to non-infected mice ($p < 0.01$), and while we found that RS appeared to result in a slight increase in the virus-specific antibody response at this time, the effect did not reach statistical significance ($p = 0.182$; Fig. 3-5A). The effects of

both infection and stress on virus-specific antibody levels were much more pronounced at day 16 p.i. than at day 8 (Fig. 3-5A; left vs. right). As expected, infected groups (I/NR, I/R) had significantly more antibody than non-infected groups (NI/NR, NI/R) ($p < 0.0001$), and infected mice had higher levels of virus specific antibody in their serum than at day 8 (Fig. 3-5A; left). Moreover, at day 16 p.i. virus specific antibody levels were significantly increased in the serum of I/R ($n = 10$) mice when compared to I/NR ($n = 10$) mice ($p < 0.05$) indicating that stress increased virus-specific antibody levels in the circulation (Fig. 3-5A; right). The effect of restraint stress on virus-specific antibody levels did not appear to be attributable to an alteration in class switching as the same trend was observed in virus specific serum IgM and IgG levels at day 8 p.i. (data not shown).

Because serum antibody levels may not be indicative of the humoral response that is occurring at the site of infection (CNS), we used ELISPOT assays to determine the effects of RS on the number of virus-specific antibody secreting cells (ASCs) in the CNS as well as the spleen at days 8 and 16p.i. No virus-specific ASCs were detected in the CNS of either infected or non-infected groups at either time point (data not shown). Conversely, small numbers of virus specific ASCs were detected from the spleens of mice (Fig. 3-5B). While the effects of infection and stress on the number of antibody secreting cells were not found to be statistically significant at either day 8 or day 16 p.i., the results appeared to mirror the findings from the ELISA, insofar as there were generally more virus specific APCs in I/R mice than I/NR mice groups at both day 8 and day 16 p.i. (Fig. 3-5B).

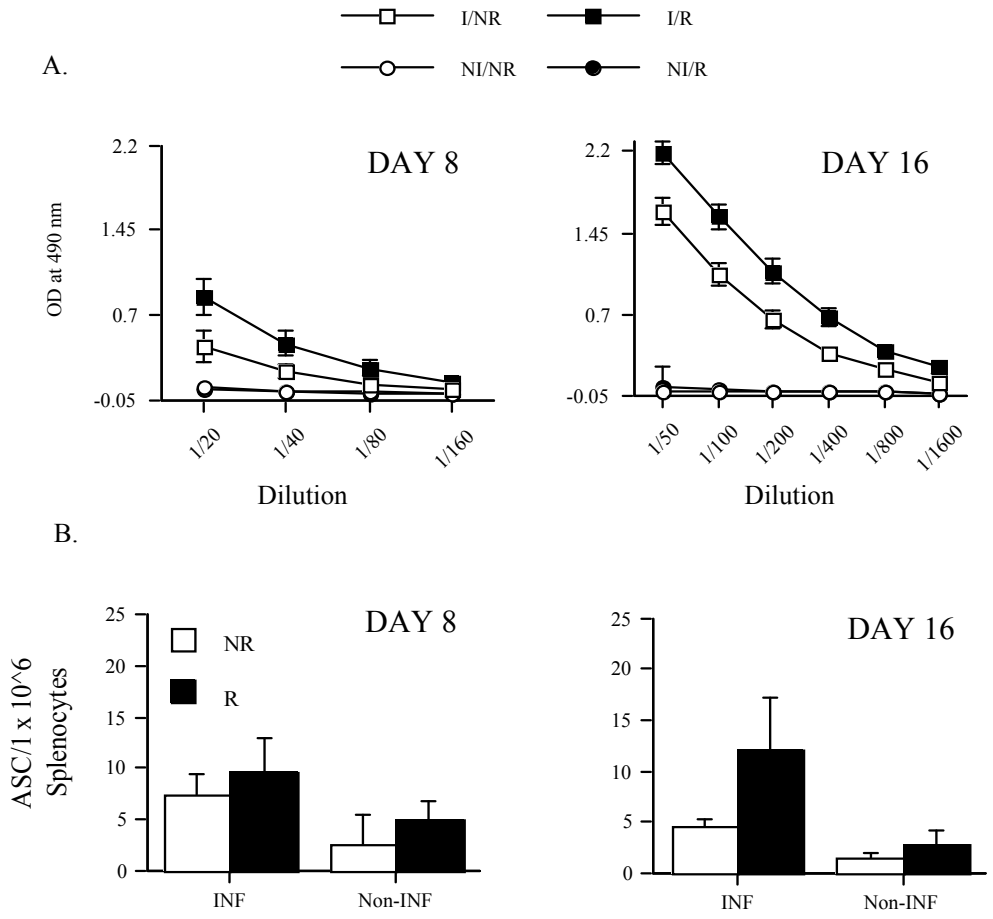


Figure 3-5. Restraint stress facilitates the production of TMEV-specific antibody response. Female SJL mice aged 4-5 weeks were grouped according to infection and restraint. Beginning at day -1 post infection (p.i.) mice in the restrained groups were subjected to 8 hours nightly restraint sessions which lasted until day 8 p.i. Serum isolated upon completion of the experiments (day 8 or day 16 p.i.) was used to test for virus-specific Ig by ELISA. A. Infected mice had elevated virus specific antibody levels at day 8 p.i. which were further increased by day 16 p.i. Stress increased the level of this response at both time points. B. ELISPOT assays were used to test for virus specific antibody producing cells in the spleens of mice at days 8 and 16 p.i. Results are combined means ± SEM of 4 separate experiments, and are representative of 6-10 mice per group.

3.2.4 Restraint stress decreases virus-specific Th1 and Th2 cytokine secretion at day 8

As our ELISPOT assays were designed to enumerate IFN-γ specific T-cells, it could have been possible that stress altered virus-specific T-cell responses by inducing a Th1/Th2 shift (Elenkov, 2004). To ascertain if such a shift occurred, we first measured serum cytokines. As seen in Table 3-2, RS caused a significant decrease in the concentrations of circulating type 1 and type 2 cytokines including IFN-γ, IL-12(p40), IL-12(p70) (type 1), IL-4, and IL-5 (type 2; $ps < 0.05$). However, infection was only found to statistically increase circulating concentrations of IL-12(p40) at this time point ($p < 0.05$) (Table 3-2).

Stress also caused a decrease in the circulating concentrations of the chemokines RANTES and MCP-1, but increased serum levels of IL-6, G-CSF and KC (Table 3-3). As observed for type 1 and type 2 cytokines these concentrations were not significantly altered by infection with TMEV, except in the case of KC, in which case infection decreased serum concentrations (Table 3-3).

Table 3-2 Stress decreases type 1 and type 2 serum cytokines

	Cytokine Concentration (pg/ml)[§]								
	Type 1					Type 2			
GROUP	IL-12(p40)[a,b,c,e,f,h]	IL-12(p70)[a,c,e]	IFN-γ[a]	IL-2	TNF-α	IL-4[a]	IL-5[a,c,e,f,h]	IL-10	IL-13
I/NR	311.47 ± 16.68	339.3 ± 39.0	231.29 ± 17.65	9.23 ± 0.88	372.51 ± 29.08	1.59 ± 0.21	8.47 ± 1.71	123.63 ± 32.0	117.78 ± 9.8
I/R	144.10 ± 17.32	194.14 ± 40.52	151.42 ± 28.71	6.39 ± 0.74	250.83 ± 39.31	1.2 ± 0.12	3.56 ± 1.42	72.18 ± 11.7	84.47 ± 14.78
NI/NR	263.17 ± 19.53	304.04 ± 34.66	221.8 ± 28.69	9.76 ± 2.53	352.46 ± 33.6	1.6 ± 0.18	9.9 ± 1.94	103.73 ± 9.91	127.86 ± 31.74
NI/R	117.14 ± 10.16	217.98 ± 37.92	155.82 ± 26.42	8.21 ± 1.50	333.60 ± 63.49	1.32 ± 0.12	4.02 ± 0.65	88.99 ± 14.67	96.77 ± 19.07

§ Values were rounded to the nearest 0.01 after statistics were analyzed

[a]Main effect of restraint; [b]Main effect of infection; [c]I/NR vs I/R; [d]I/NR vs NI/NR; [e]I/NR vs NI/R; [f]I/R vs NI/NR; [g]I/R vs NI/R; [h]NI/NR vs NI/R (ps < 0.05)

Results are means ± SEM from 3 independent experiments

n = 5-7; N = 24

Table 3-3 Effects of stress on serum chemokines and hematopoietic factors

	Concentration (pg/ml)[§]							
	Chemokines					Hematopoietic Factors		
GROUP	MCP-1[a,c,f]	MIP-1α	MIP-1β	RANTES[a,c,f,g]	EOTAXIN	KC[a,b,c,e,f,g,h]	G-CSF[a,e,h]	IL-6[a,e,g,h]
I/NR	293.72 ± 15.83	418.92 ± 24.12	49.33 ± 3.01	158.933 ± 12.82	126.78 ± 33.9	38.54 ± 3.87	39.25 ± 4.82	24.67 ± 1.3
I/R	213.72 ± 28.94	348.25 ± 25.57	35.09 ± 5.56	113. 25 ± 8.13	77.84 ± 25.49	78.08 ± 9.64	407.29 ± 159.06	28.14 ± 4.14
NI/NR	295.07 ± 34.5	424.82 ± 18.27	44.03 ± 5.87	149.82 ± 5.93	166.57 ± 60.58	39.83 ± 3.867	43.14 ± 5.21	23.13 ± 2.44
NI/R	229.567 ± 21.8	380.81 ± 47.29	42.23 ± 5.41	146. 92 ± 9.16	115.43 ± 52.93	133.01 ± 20.34	711.08 ± 308.7	38.78 ± 2.49

§ Values were rounded to the nearest 0.01 after statistics were analyized

[a]Main effect of restraint; [b]Main effect of infection; [c]I/NR vs I/R; [d]I/NR vs NI/NR; [e]I/NR vs NI/R; [f]I/R vs NI/NR; [g]I/R vs NI/R; [h]NI/NR vs I/R (ps < 0.05)

Results are means ± SEM from 3 independent experiments

n = 5-7; N = 24

Isolation of mRNA from unstimulated splenocytes yielded similar results as those obtained with cytokines, insofar as stress appeared to decrease levels of both IFN-γ as well as IL-5, and did not support the occurrence of a Th1 to Th2 shift (Fig. 3-6A-C).

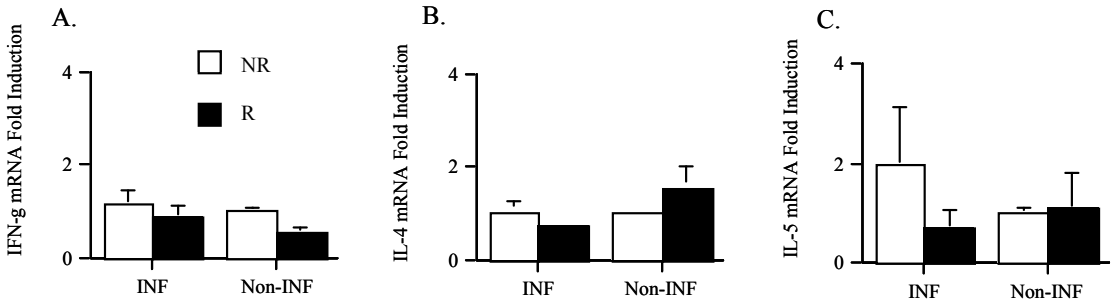


Figure 3-6. Neither infection nor stress significantly altered mRNA levels of cytokines from unstimulated splenocytes at day 8 p.i. Mice were assigned to I/NR, I/R, NI/NR and NI/R groups. Stress sessions began at day -1 p.i. and lasted until day 8 p.i. At day 8 p.i. total RNA was isolated from splenocytes (1.0×10^7). A-C. cDNA was generated using random primers and RT-PCR was performed using syber green and primers specific for IFN-γ (A), IL-4 (B), and IL-5 (C). Samples were first normalized to β-actin, then normalized to NI/NR group.

Therefore, to more specifically address the effects of RS on helper T-cell responses, we repeated the above experiments and isolated cells from the CNS and spleens of mice at day 8 p.i. CNS-IL as well as spleen cells were stimulated either with plate bound anti-CD3 and anti-CD28, whole UV-inactivated TMEV (UV-BeAn), or media for 72 hours and supernatants were measured for Th1, Th2 and Th17 cytokines. CNS-IL from I/NR mice appeared to produce more IFN-γ when stimulated with antibodies to both CD3 and CD28 than any other group, possibly indicating a higher

number of Th1 clones (Fig. 3-7A). When stimulated with UV-BeAn, CNS-IL from I/NR mice produced statistically more IFN-γ than CNS-IL isolated from I/R mice (Student's *t*-test; $p < 0.05$; Fig. 3-7A). I/NR mice secreted significantly higher levels of IL-17A, a marker of Th17 cell effector function, when stimulated with UV-BeAn, than I/R (Student's *t*-test; $p < 0.05$; Fig. 3-7B). Moreover, I/NR mice were also found to produce statistically more IL-2 when stimulated with UV-BeAn than I/R mice (Fig. 3-7C; Student's *t*-test; $p < 0.05$). Generally, the same trend existed for the Th2 cytokines IL-5 and IL-10, when stimulated with either anti-CD3 and anti-CD28 antibodies or UV-BeAn, but these effects were not statistically significant (data not shown). These results indicate that at day 8 p.i., infected mice possess increased levels of virus-specific pro-inflammatory T-cells within the CNS, and that stress decreases these responses.

In the spleen, stressed mice (I/R, NI/R) groups appeared to have fewer Th1 and Th2 clones when compared to their non-stressed counterparts (I/NR, NI/R) as indicated by a general reduction in IFN-γ, IL-4 and IL-10 secretion following anti-CD3 and anti-CD28 stimulation (Fig. 3-8A-B, D). Importantly, infected animals produced more IFN-γ, Il-17A, IL-4 and IL-10 following stimulation with UV-BeAn than non-infected mice ($ps < 0.05$). With the exception of IL-17A, comparisons between I/NR and I/R mice groups confirmed that stress decreased these virus specific immune responses ($ps < 0.05$). Taken together these results suggest that restraint stress administered during acute infection with TMEV inhibits virus-specific Th1 and Th2 cytokine production from cells isolated from both the periphery as well as from the CNS (Figs. 3-7 and 3-8).

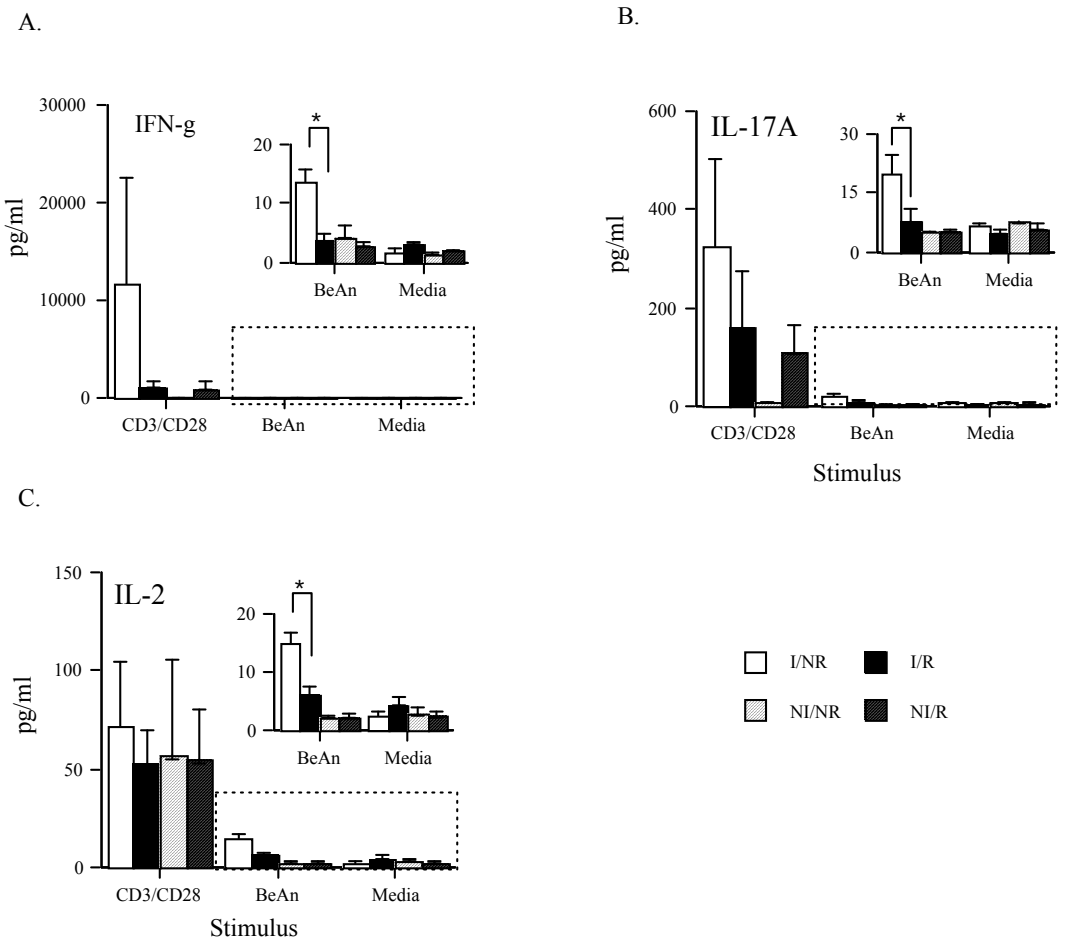


Figure 3-7. RS decreases virus-specific proinflammatory responses occurring in the CNS at day 8 p.i. with TMEV. CNS-IL were extracted from mice at day 8 p.i. CNS-IL (5.0×10^4) were combined with 1.0×10^6 sygeneic irradiated feeders and cultured with plate-bound α-CD3 (10μg/ml) and α-CD28 (2μg/ml), purified UV-inactivated BeAn, or media for 72 hours. Supernatants were used to measure cytokine production by luminex or ELISA. A-C. I/NR mice produced increased amounts of the pro-inflammatory cytokines IFN-γ (A), IL-17A (B) and IL-2 (C) when re-stimulated with whole UV-BeAn compared to media controls. I/R mice produced significantly less of these cytokines than infected/non-stressed mice. Significant effects are shown. Results are combined means ± SEM of 3 separate experiments, and are representative of 4-6 mice per group. Student's *t*-tests were used to test significance. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

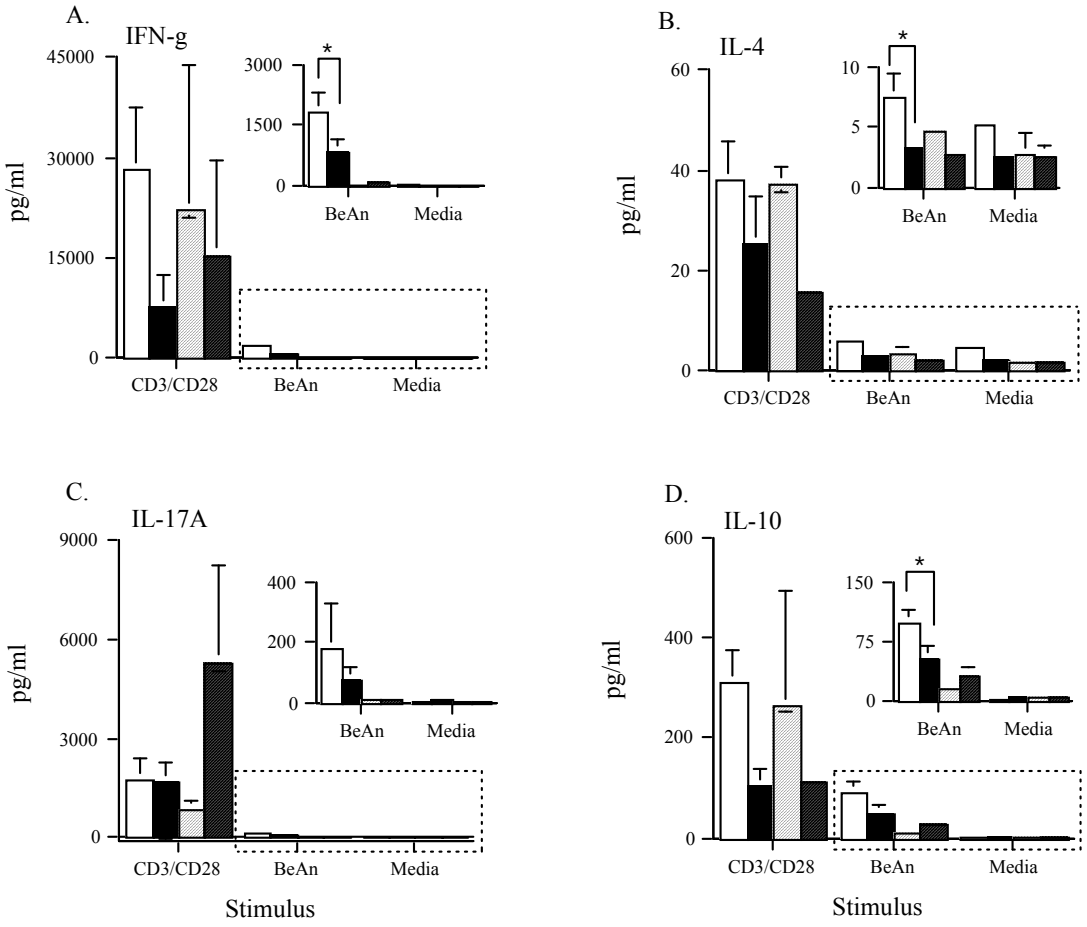


Figure 3-8. Restraint stress decreases both virus-specific pro-inflammatory and anti-inflammatory responses occurring in the spleen at day 8 p.i. with TMEV. Splenocytes (1.0 x 10^6) were isolated from I/NR, I/R, NI/NR, NI/R mice at day 8 p.i. and cultured with plate-bound α-CD3 (10μg/ml) and α-CD28 (2μg/ml), purified UV-inactivated BeAn (1.0 μg), or media for 72 hours at 37 C and 5.0% CO_2. Supernatants were used to measure cytokine production by luminex or ELISA. A-D. Splenocytes produced increased amounts of the pro-inflammatory cytokines IFN- γ (A), IL-17A (C) and IL-4 (B) and IL-10 (D) when re-stimulated with whole UV-BeAn compared to media controls.

To explore the effects of stress on type 1 and type 2 responses further, we questioned whether stress altered the polarization of T-cells. We hypothesized that if RS caused Th1 and Th2 immunosuppression rather than just decreased cytokine secretion, then T-bet and GATA-3, the transcription factors for Th1 and Th2 polarization respectively, would also be decreased. Therefore, we first measured mRNA levels of both T-bet and GATA-3 by quantitative RT-PCR from RNA isolated from unstimulated splenocytes. We found that at day 8 p.i., mice in the I/NR group had significantly increased mRNA levels of T-bet ($\underline{p} < 0.05$), when compared to other groups, and that RS significantly decreased mRNA levels of T-bet (Fig. 3-9A, top; $\underline{p} < 0.01$). Although not as robust, the same effects of infection and stress were demonstrated for GATA-3 mRNA levels, such that the I/NR group possessed higher expression levels of GATA-3 than any other group, and that RS significantly decreased GATA-3 expression (Fig 3-9A, bottom; $\underline{p}$'s < 0.05). To determine if these effects of stress indeed translated into decreased protein levels, and to ensure the validity of our findings, we repeated the experiment, but measured T-bet and GATA-3 protein levels by immunoblots from nuclear extracts of splenocytes at day 8 p.i., following stimulation plate-bound anti-CD3 and anti-CD28 antibodies, $VP2_{74\text{-}86}$, $VP3_{159\text{-}166}$ or NS peptides for 5 and 24 hours. Regardless of the stimulus, prior infection with TMEV increased only T-bet relative to β-actin controls in non-stressed mice after 5 hours of stimulation (Fig. 3-9B; top). Contrary to the results demonstrated by RT-PCR, GATA-3 nuclear protein levels were not affected by RS (Fig. 3-9B; top). While not as pronounced, the same trend existed following 24 hours of stimulation (Fig. 3-9; bottom). Densitometry quantification of

protein levels at 5 hours of stimulation resembled results obtained from mRNA fold induction by RT-PCR, insofar as stress was appeared to decrease T-bet nuclear protein levels, but did not appear to have influenced GATA-3 protein (Fig. 3-9C).

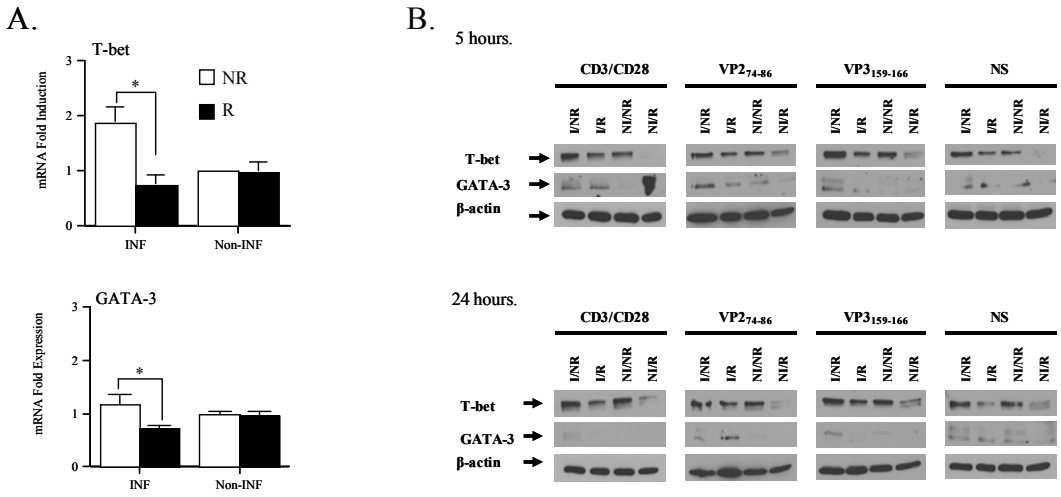
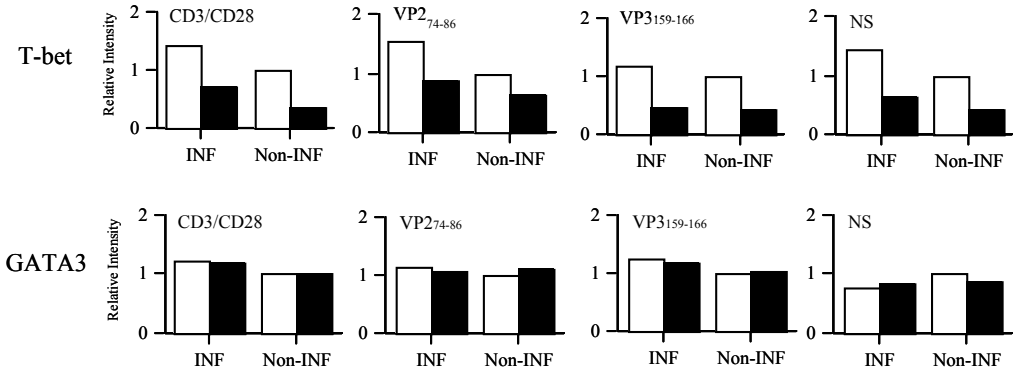


Figure 3-9. Restraint stress decreased T-bet, and causes decreased virus specific IFN-γ secretion from stimulated splenocytes isolated at day 8 p.i. A. RNA isolated from splenocytes of mice at day 8 p.i. was reversed transcribed into cDNA. mRNA expression of T-bet and GATA-3 tested by RT-PCR. Data represent fold induction relative to β-actin and normalize to NI/NR group. B. In separate experiments splenocytes (1.0×10^6) were cultured with plate-bound α-CD3 (10μg/ml) and α-CD28 (2μg/ml), $VP2_{74-86}$ (2.0 μM), $VP3_{155-166}$ (2.0 μM), or non-specific peptide (NS; 2.0 μM) or for 5 or 24 h at 37°C and 5.0% CO_2. Nuclear extracts were subjected to immunoblots specific for T-bet (B; top row); or GATA-3 (B; middle row) or β-actin (B; bottom row). C. Densitometric analysis at of T-bet relative to β-actin was normalized to the NI/NR group at 5 h stimulation. D. Supernatants were used to measure IFN-γ production by ELISA. A and D results represent means ± SEM of 3 separate experiments comprising 5-7 mice per group. B represent pooled samples from 2 mice per group; this experiment was repeated with similar results (not shown). C are mean densitometric analysis of results obtained of both experiments in B at 5 hours stimulation and represent 4 mice per group. * $P < .05$.

C.



D.

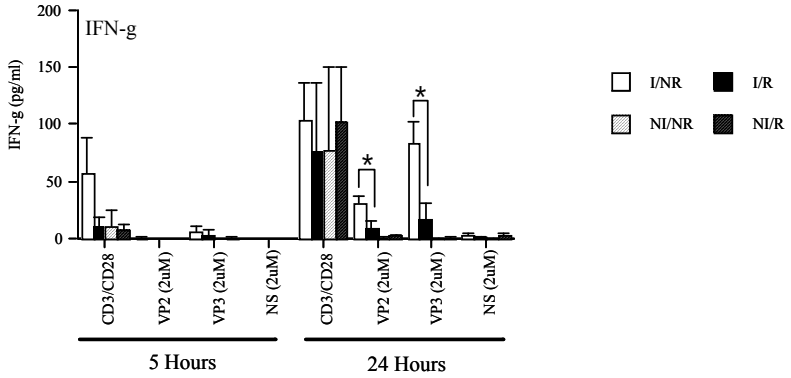


Figure 3-9, continued.

In order to determine if these effects of stress correlated with decreases in IFN-γ, a known target of T-bet, we measured IFN-γ secretion from previously stimulated splenocyte cultures taken from infected (I/NR, I/R), or non-infected (NI/NR, NI/R) groups at day 8 p.i. Figure 3-9D shows that infection resulted in increased IFN-γ production by 24 hours following stimulation anti-CD3 and anti-CD28, or viral peptides ($VP2_{74-86}$, $VP3_{159-166}$), but not the NS peptide. Restraint stress was found to significantly decrease both virus-specific CD4 ($VP2_{74-86}$) and CD8 ($VP3_{159-166}$) responses by

approximately 21% and 66% respectively (Students *t*-test; ps < 0.05). These results are highly reminiscent of our ELISPOT data at this time, and indicate that RS results in a decrease in IFN-γ secretion as well as the number of IFN-γ producing cells (Fig. 3-4).

3.2.5 Restraint stress modestly alters $CD4^+$ and $CD8^+$ T-cell percentages at days 8 and 16 p.i.

The possibility that RS was mediating its actions in the periphery (spleen) by decreasing the total percentages of $CD4^+$ and $CD8^+$ T-cells, either by trafficking or cell death, could account for our observed reductions in T-cell responses. In order to investigate this possibility, we measured T-cell percentages in isolated spenocytes from I/NR and I/R mice by flow cytometry at days 8 and 16 p.i. At day 8 p.i. we found that RS resulted in a very slight (64.5% vs. 62%), but highly significant (Student's *t* test; $p < 0.01$), decrease in the percentage of $CD4^+$ T-cells (Fig. 3-10A). The effect of RS on $CD4^+$ percentage was found to persist until day 16 p.i. (63.5% vs. 57.5%) despite the secession of RS (Student's *t* test; $p < 0.001$).

The percentages of $CD8^+$ T-cells in the spleens were higher at day 8 than day 16 p.i. In contrast to that observed for $CD4^+$ T-cells, RS resulted in a moderate, but highly significant increase in the percentages of $CD8^+$ T-cells at both day 8 (31% vs. 35%) and day 16 (20% vs. 25%) (Fig. 3-10B; Student's *t* test; ps < 0.01). These results indicate that RS is capable of modifying peripheral $CD4^+$ and $CD8^+$ T-cell percentages. However, while statistically significant, these effects account for up to only 5% alteration, and thus are unlikely to fully account for our observed RS-induced

immunosuppression of virus specific $CD4^+$ ($VP2_{74-86}$) effector function. Additionally, as stress resulted in an increased percentage of $CD8^+$ T-cells, the observed decrease in virus-specific $CD8^+$ restricted ($VP3_{155-169}$) responses becomes more significant.

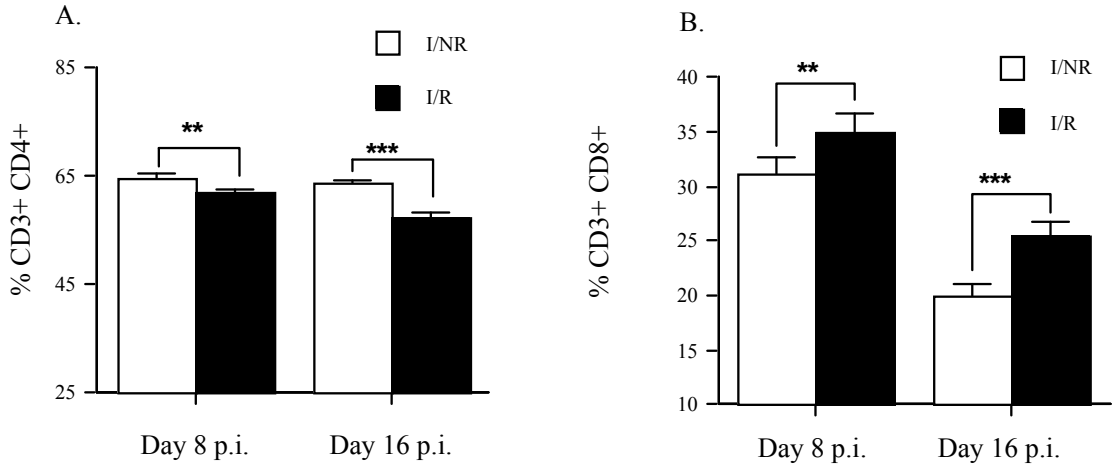


Figure 3-10. Restraint stress differentially alters $CD4^+$ and $CD8^+$ T-cell percentages at days 8 and 16 p.i. Female SJL mice aged 4-5 weeks were assigned to either infected/non-restrained (I/NR), or infected/restrained (I/R), groups. Beginning at day -1 post infection (p.i.) mice in the restrained groups were subjected to 8 hours nightly restraint sessions which lasted until day 8 p.i. At day 8 or 16 p.i. percentages of $CD4^+$ (A) and $CD8^+$ (B) T-cells from isolated splenocytes (1.0×10^6) was determined by flow cytometry. For each time point n = 7 per group.
Results are means ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$.

3.2.6 Effects of glucocorticoids on adaptive immune responses to TMEV at day 8 p.i.

As restraint stress has been previously demonstrated to increase serum corticosterone in SJL mice infected with TMEV (Sieve et al., 2004), glucocorticoids could be responsible for our observed immunosuppression. To test the hypothesis that glucocorticoids mediate stress-induced immunosuppression, we infected mice and treated them with dexamethasone (DEX), RU486 or respective vehicle controls, and measured virus-specific immune responses during their peak at day 8 p.i. Analysis of change in body weight percentages indicated that our treatments were successful because as seen before, (Fig. 3-4B) stress caused a decrease in body weight which was reversed by concurrent administration of RU486 (Fig. 3-11A). At the same time administration of DEX mimicked the effect of RS (Fig. 3-11A). The effects of these treatments became statistically significant by day 4 p.i. Additionally, we found that the total number of isolated splenocytes were still reduced in stressed mice treated the RU486 (Fig. 3-11B). In contrast, the effects of RS on T-cell percentages in the spleens were entirely mediated by glucocorticoids as these effects were completely reversed when RU486 was aministered to I/R mice, but were reestablished in I/NR mice when administered DEX (Fig. 3-12A-B). As before, stress decreased the number of $VP2_{74-86}$ and $VP3_{155-169}$ specific IFN-γ producing cells in the spleen, and DEX treatment mimicked these effects (Fig. 3-12B-C). However, unlike T-cell percentages, RU486 treatment during concurrent RS did not completely alleviate the effects of RS (Fig. 3-12D). In agreement with our previous data, almost no $VP2_{74-86}$ reactive IFN-γ producing cells were detected in the CNS of infected animals, while a robust $VP3_{155-169}$ response was evident (Fig. 3-

12E). Compared to the spleen, the responses in the CNS exhibited similar effects of RU486 and DEX treatments on the number of $VP3_{155-169}$ reactive IFN-γ producing cells although the effects failed to reach statistical significance; possibly attributable to a low sample number (n = 4 per group; Fig. 3-12F).

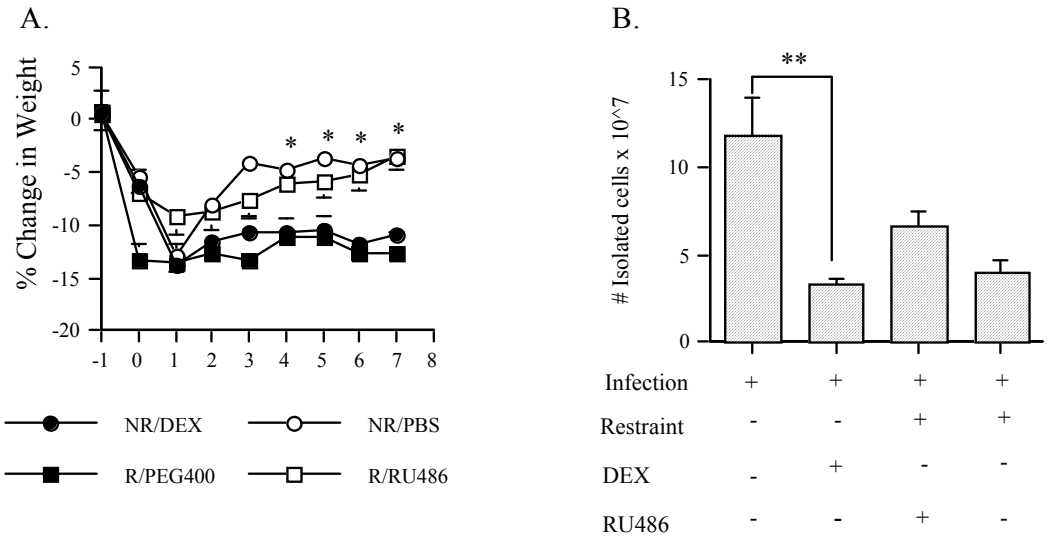


Figure 3-11. Restraint stress induced weight loss is mediated by the actions of glucocorticoids. Female SJL mice aged 4-5 weeks were assigned to either infected/non-restrained PBS treated (I/NR-PBS), infected/non-restrained DEX treated (I/NR-DEX), infected/restrained PEG treated (I/R-PEG), or infected/restrained RU486 treated (I/R-RU486). Beginning at day -1 post infection (p.i.) mice in the restrained groups were subjected to 8 hours nightly restraint sessions which lasted until day 8 p.i. Treatments with DEX (1.0 mg/kg) and RU486 (25mg/kg) or vehicle control (PBS or PEG400 respectively) were administered nightly 1 h prior to each stress session by i.p. or s.c. injection in a total volume of 0.1ml. A. Body weight was recorded nightly before injections and is presented as percent change from baseline (day -1 p.i.). B. At the termination of the experiment the total number of spleen cells were determined by trypan blue exclusion. Results are combined means ± SEM, of at least 2 separate experiments (n = 5-10 mice per group).

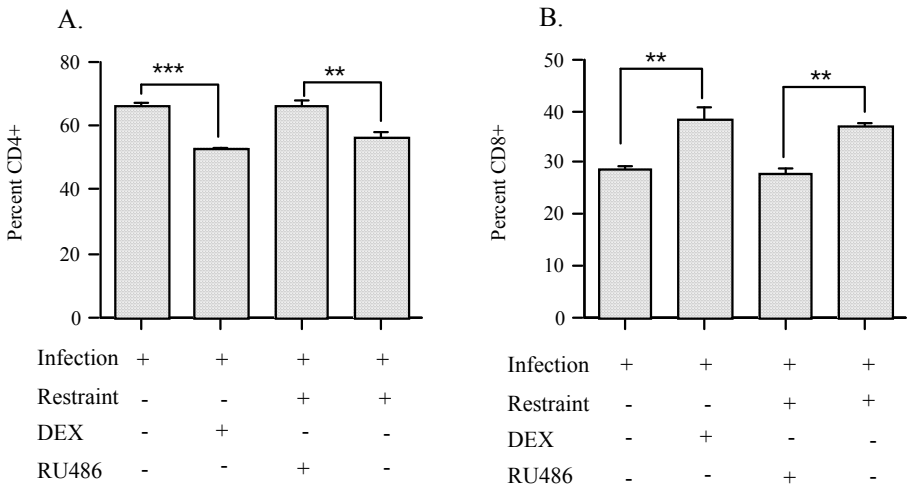


Figure 3-12. Effects of RS on T-cell number and virus-specific effector function are partly due to the actions of glucocorticoids. Female SJL mice aged 4-5 weeks were assigned to either infected/non-restrained PBS treated (I/NR-PBS), infected/non-restrained DEX treated (I/NR-DEX), infected/restrained PEG treated (I/R-PEG), or infected/restrained RU486 treated (I/R-RU486). Beginning at day -1 post infection (p.i.), mice in the restrained groups were subjected to 8 hours nightly restraint sessions which lasted until day 8 p.i. Treatments with DEX (1.0 mg/kg) and RU486 (25mg/kg) or vehicle control (PBS or PEG400 respectively) were administered nightly 1 h prior to each stress session by i.p. or s.c. injection in a total volume of 0.1ml. A. Effects of glucocorticoids on percentage of $CD4^+$ and $CD8^+$ (B) cells in the spleens of infected mice. C. The effects of RS, DEX and RU486 treatment on the number of IFN-γ producing $CD4^+$ restricted ($VP2_{74-86}$) cells in the spleen. D. The effects of RS, DEX and RU486 treatment on the number of IFN-γ producing $CD8^+$ restricted ($VP3_{155-169}$) cells in the spleens. E. The effects of RS, DEX and RU486 treatment on the number of IFN-γ producing $CD4^+$ restricted ($VP2_{74-86}$) cells in the CNS. F. The effects of RS, DEX and RU486 treatment on the numbers of CD8 restricted ($VP3_{155-169}$) IFN-γ producing cells in the CNS. Results are combined means ± SEM of 2-5 independent experiments.

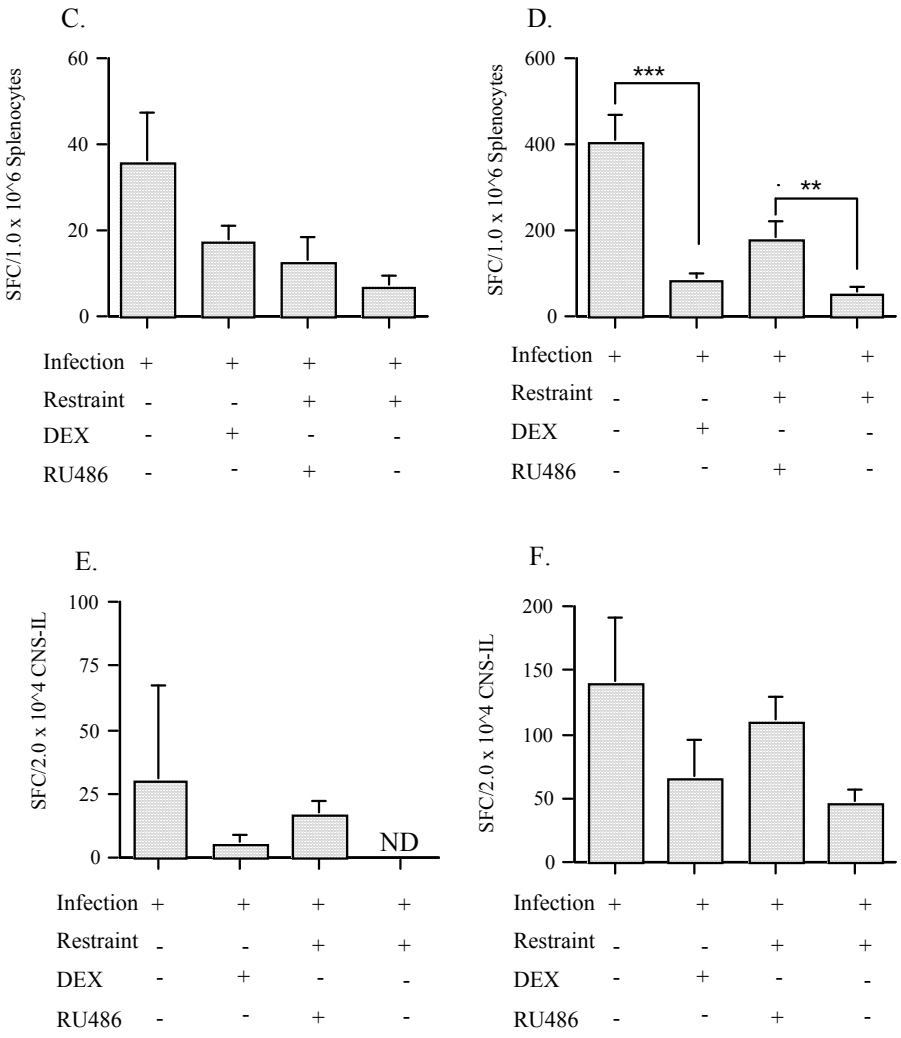


Figure 3-12, continued.

Unlike T-cell effector function, the effects of glucocorticoids on serum antibody levels were not as straightforward. At day 8 p.i., DEX treatment appeared to reduce serum antibody levels in I/NR mice (Fig. 3-13), indicating a potential role for glucocorticoids in inhibiting antibody levels. I/R mice treated with the glucocorticoid receptor antagonist RU486 also appeared in decreased TMEV specific antibody levels

(Fig. 3-13). However, as seen in Figure 3-5 these effects at this timepoint were not significant.

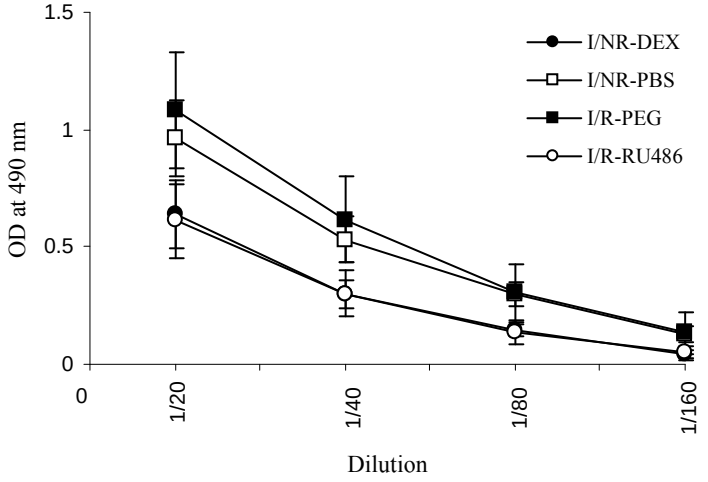


Figure 3-13. Restraint stress induced increase in TMEV-specific serum antibody levels does not appear to be attributable to the actions of glucocorticoids. Mice were assigned to either infected/non-restrained PBS treated (I/NR-PBS), infected/non-restrained DEX treated (I/NR-DEX), infected/restrained PEG treated (I/R-PEG), or infected/restrained RU486 treated (I/R-RU486). At day -1p.i. mice in the restrained groups were subjected to 8 h nightly restraint sessions which lasted until day 8 p.i. Treatments with DEX (1.0 mg/kg), RU486 (25mg/kg) or vehicle control (PBS or PEG400 respectively) were given nightly 1 h prior to each stress session by i.p. or s.c. injection in a total volume of 0.1ml. Virus-specific antibody levels were measured from serum taken at day 8 p.i. by ELISA. Results are means ± SEM of 10 mice per group from 2 separate experiments.

3.2.7 Effect of glucocorticoids on the functions of IFN-γ production

In the above experiments glucocorticoid-induced immunosuppression could have been brought about by suppressing the function of antigen presenting cells, essentially inhibiting the generation of TMEV-specific immunity. To more closely examine the effects of glucocorticoids on the effector functions in T-cells, we first stimulated splenocytes isolated from I/NR mice with $VP2_{74-86}$, $VP3_{155-169}$, or NS peptides in the presence of varying concentrations of DEX for 24 hours, and then determined IFN-γ concentrations from the culture supernatants. DEX treatment of cells resulted in a dose dependent reduction in IFN-γ production for both $VP2_{74-86}$, and $VP3_{155-169}$ stimulated splenocytes (Fig. 3-14A), indicating that either IFN-γ production is inhibited by DEX treatment or that DEX treatment causes cell death. To investigate the latter possibility we stimulated splenocytes isolated from a I/NR mouse with either viral peptides, control peptides, media alone or varying concentrations of DEX. Utilizing an MTT assay, the viability of the splenocytes were determined at 24 hours post stimulation. Increasing concentrations of DEX resulted in a dose dependent reduction of viability (Fig. 3-14B). At 100nM concentration of DEX there was approximately 32% reduction in viability. While it seems unlikely that this reduction in viability reflected exclusive death of IFN-γ production T-cells in our culture system, we cannot rule out this possibility. Taken together, these results may support a role for glucocorticoid induced inhibition of TMEV specific T-cell responses.

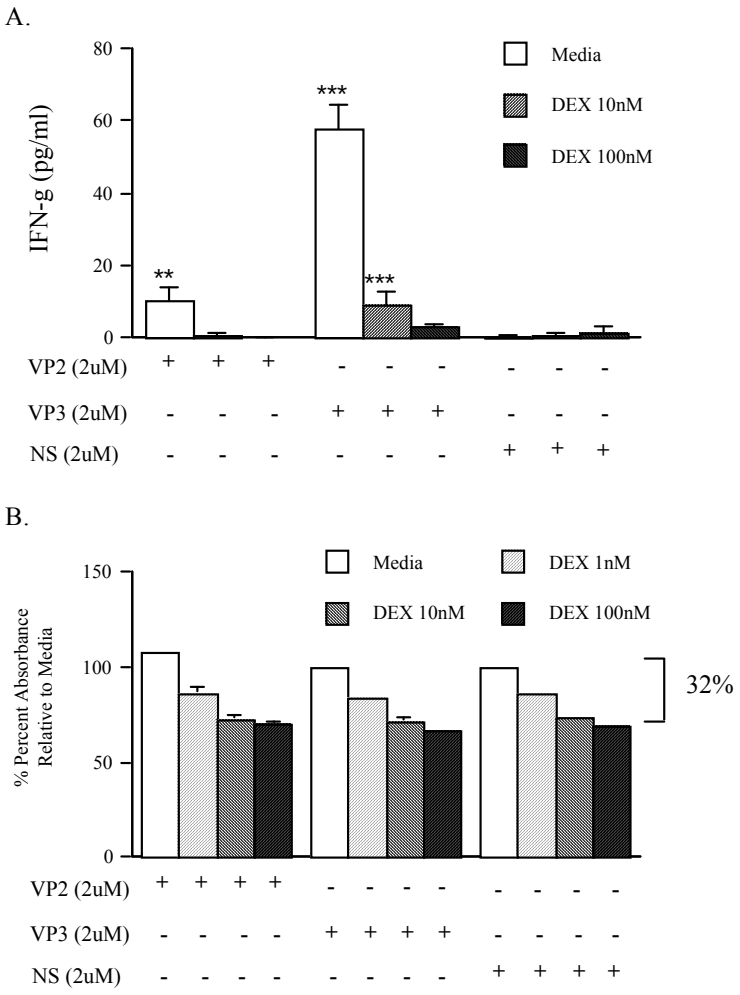


Figure 3-14. Glucocorticoids decrease virus specific IFN-γ production. Non-restrained female SJL mice (n = 2) were infected with TMEV. A. At day 8 p.i. 1.0 x 10^6 spleen cells were stimulated with 2μM $VP2_{74-86}$ (VP2), $VP3_{155-169}$ (VP3) or non-specific (NS) peptide for 24 h in presents of varying concentrations of dexamethasone (DEX) or media. Concentrations of IFN-γ from supernatants were determined by ELISA. B. At day 8 p.i. splenocytes from a I/NR SJL mouse were isolated and simulated as described. After 24 h of stimulation MTT was used to check viability of cells. Samples from all conditions were run in duplicate with the exception of the NS stimulated cells from the MTT assay (B), which is representative of only one sample per condition. Results are combined means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

4. Discussion

In multiple sclerosis, the production of proinflammatory cytokines, particularly IFN-γ, within the CNS by $CD4^+$ or $CD8^+$ T-cells is generally perceived as a negative event (Sospedra and Martin, 2005). In fact, when administered as a therapy to MS patients, IFN-γ was associated with increased frequency of relapses (Panitch et al., 1987). Experimentally, IFN-γ has been shown to directly induce neurotoxicity by resulting in the phosphorylation of the AMPA receptor GLuR1, a process dependent on the direct association with the IFN-γR and its activation (Mizuno et al., 2008). In addition, it has been demonstrated that induced IFN-γ expression within the CNS can inhibit re-myelination by a process dependent on ER stress (Lin et al., 2006). IFN-γ may also modulate MS through its ability to induce MHC class II expression on astrocytes (Wong et al., 1984) as well as through its ability to induce MHC class I expression on electrically silent neurons (Neumann et al., 1995). The later would expose neurons to a potential cytolytic attack by $CD8^+$ T-cells, which have been shown to be oligoclonally expanded in MS patients and outnumber $CD4^+$ T-cells within the lesions by 3:1 (Bebbe et al., 2000). In support of the deleterious effects of type 1 immune responses in MS, T-bet and phosphorylated STAT1 levels have recently been shown to be elevated in peripheral blood mononuclear cells taken from patients during relapses compared to those in remission or healthy controls. Furthermore, treatment with oral glucocorticoids inhibited this effect (Frisullo et al., 2007).

Another proinflammatory cytokine that has recently received considerable attention in the realm of autoimmune diseases is IL-17. While T-cells and neutrophils

are capable of producting IL-17, the major source of IL-17 is the Th17 cell, which in humans have been shown to also be capable of producing IFN-γ (Annunziato et al., 2007). Supporting a role for Th17 cells in the pathogenesis of MS, patients have increased IL-17 mRNA expression in their brain and CSF (Witowskiet et al., 2004). Additionally, Vaknin-Dembinsky et al., (2006), have demonstrated that IL-23, a cytokine responsible for Th17 cell proliferation, is increased in dendritic cells isolated from MS patients, and that isolated $CD4^+$ T-cells stimulated with α-CD3 antibodies produce more IL-17 than healthy controls (2006). Mechanistically, it is thought that these Th17 polarizing dendritic cells arise from the differentiation of circulating monocytes by activated cerebrovascular endothelial cells at the site of the lesion (Ifergan et al., 2007). Finally, involvement of Th17 cells in the pathogenesis of MS is supported by the findings that IL-17R is expressed on human endothelial cells of MS patients but not patients with other neurological diseases; that IL-17 is capable of incurring BBB breakdown and Th17 cells can kill neurons in a granzyme dependent fashion (Kebir et al., 2007).

Even though proinflammatory cytokine secretion can have devastating effects within the CNS, animal models of MS have shown that proinflammatory cytokine secretion can potentiate some protection. For instance, EAE is exacerbated in IFN-$\gamma^{-/-}$ mice (Krakowski and Owens, 1996; Ferber et al., 1996; Willenborg et al., 1996). In Theiler's virus infection, IFN-γ is thought to assume a protective role. During early infection it is beneficial as illustrated by the fact that it has potent *in vitro* anti-viral activity against TMEV (Welsh et al., 1995) and has been demonstrated to be required

for neuronal viral clearance from the CNS, as IFN-$\gamma^{-/-}$ mice display decreased survival rates following infection with TMEV (Rodriguez et al., 2003; Murray et al., 2002). Additionally, both IFN-$\gamma^{-/-}$ and IFN-$\gamma R^{-/-}$ mice on a normally demyelination-resistant background develop severe demyelination (Rodriguez et al., 1995; Johnston et al., 2001; Murray et al., 2002). While many studies have investigated the role of IFN-γ in TMEV infection, to date, virtually nothing is known about the involvement of Th17 cells in the pathogenesis of Theiler's virus infection. However, some studies indirectly implicated these cells in the disease process. For instance, deletion of IL-12(p40), a subunit required for the formation of both active IL-12 and IL-23 heterodimers, by antibody administration given during early infection with TMEV delays the onset of demyelination in susceptible mice (Inoue et al., 1998). In constrast, deletion of STAT4, a transcription factor responsible for IFN-γ transcription, in mice on a normally TMEV-IDD resistant background, are rendered susceptible to demyelination (Rodriguez et al., 2006). Also, the TMEV susceptibility gene *tmevp3* is suspected of coding for IL-22, an additional product of Th17 cells (Liang et al., 2006), and that this loci limits viral titers within the CNS controlling mortality due to encephalitis in SJL mice during early infection (Levillayer et al., 2007).

In the current study re-stimulation with either the immunodominant $CD4^+$ ($VP2_{74-86}$) or $CD8^+$ ($VP3_{155-169}$) peptides demonstrated that RS severely impairs the number of virus specific IFN-γ producing T-cells in the periphery, as well as the number of virus specific IFN-γ producing $CD8^+$ cells in the CNS of infected animals. Moreover, the absence of the type 1 immune response in the serum, periphery, and CNS was

coupled with reductions in virus specific type 2 responses. In isolated splenocytes Th1 and Th2 cytokine reduction was mirrored by decreases in T-bet at the mRNA and protein levels, as well as a slight decrease in GATA-3 mRNA, strongly suggesting a globalized suppressive response to stress. As such, our results are supported by those of Dobbs et al., (1996) which show that RS causes a globalized immunosuppression during acute viral infection. Additionally, we have, to our knowledge demonstrated for the first time, a suppressive effect of stress on virus specific IL-17 production within the CNS; which interestingly were not duplicated in the periphery, a finding that is currently under investigation. In contrast, the numbers of virus-specific $CD8^+$ T-cells as determined by IFN-γ ELISPOTs were correlated between the CNS and spleens of infected mice, and cytokine profiles exhibited following stimulation of CNS-ILs or splenocytes with UV-inactivated virus were in agreement, which may indicate the development of virus-specific immunity in the periphery and subsequent trafficking to the CNS. Alternatively, reductions in virus-specific responses by stress could have been brought about by reductions in lymophocyte proliferation, increased cell death within both the spleen and CNS, or enhanced efflux of cells from the CNS back into the periphery, mechanisms that are currently being investigated. Moreover, glucocorticoids were found to contribute to this RS-induced suppression as the antagonist RU486 resulted in partial restoration of T-cell effector function in both the CNS and the spleen, whereas administration of the synthetic glucococorticoid, dexamethasone mimicked the effects of stress.

The observed suppressive effects of stress and glucocorticoids on T-cell effector function, particularly virus-specific IFN-γ producing cells, could have been a byproduct

of suppressive effects directed toward antigen presenting cells, especially macrophages and dendritic cells, resulting in decreased numbers of virus specific T-cell clones. This possibility is supported by the fact that corticosterone can inhibit processing and presentation of MHC class I molecules on DCs (Truckenmiller et al., 2005), as well as decrease CD80/CD86 transcription, cause MHC class II retention and reduce IL-6, IL-12 and TNF-α production in DCs activated with LPS (Elftman et al., 2007). In support of this mechanism of immunosuppression, T-cells from stressed mice stimulated with α-CD3/ α-CD28 antibodies tended to produce less type 1 and type2 cytokines than T-cells from non-stressed mice, a finding that may indicate an overall reduction on the number of Th1/Tc1 and Th2/Tc2 clones present within the CNS and the spleen (Figs. 3-4 and 3-5). Alternatively, the increase in corticosterone by stress in our model (Sieve et al., 2004) could also act directly at the level of the T-cell by inhibiting the functions of the transcription factors responsible for mediating effector function. To date, glucocorticoids have been shown to inhibit the actions of AP-1, NFkB and GATA-3 (reviewed in Webster et al., 2002; Jee et al., 2005). Recently, the actions of glucocorticoids, specifically dexamethasone, have been demonstrated to inhibit the binding of T-bet to the IFN-γ promoter, possibly providing a mechanism for defective IFN-γ transcription and production in previously polarized Th1 or Tc1 effector T-cells (Liberman et al., 2007).

The transcription factor T-box expressed in T-cells (T-bet) brings about polarization of naive Th0 cells to the functional Th1 phenotype, and is required for optimal trafficking of this subtype into inflamed tissue (Szabo et al., 2000; Lord et al.,

2005). Additionally, T-bet has been implicated in the development of functionally active NK cells (Szabo et al., 2002) and NKT cells (Townsend et al., 2004), as well as the optimal production of IFN-γ from dendritic cells (Lugo-Villarino et al., 2003). T-bet is also reported to play a role in class switching in B-cells (Peng et al., 2002; Xu and Zhang, 2005). Early *in vitro* experiments indicated that T-bet was not solely responsible for the production of IFN-γ production by $CD8^+$ T-cells (Szabo et al., 2002), which was later shown to be under the cooperative control of another T-box transcription factor, Eomesodermin (Eomes; Pearce et al., 2003). However, more recent studies indicate that T-bet is important for antigen specific cytotoxicity in $CD8^+$ T-cells (Sullivan et al., 2003), and has most recently been shown to be required for the secretion of IFN-γ from $CD8^+$ T-cells during infection (Mayer et al., 2008). Therefore, it is not surprising that T-$bet^{-/-}$ mice have been shown to be more susceptible to HSV-2 and Vaccinia Virus (Svensson et al., 2005; Matsui et al., 2005) infections than wild-type controls. Interestingly, the immunologic phenotype of restraint stressed mice compares with that of the T-$bet^{-/-}$, as RS mice are known to display reduced NK cell function (Welsh et al., 2004; Teng et al., 2005), decreased $CD4^+$ and $CD8^+$ T-cell responses to viral infections (Sheridan et al., 1991; Dobbs et al., 1996; Freeman et al., 2007; current study), decreased T-cell trafficking (Zhang et al., 1998; Ottaway and Husband, 1994; Mizobe et al., 1997), and display greater susceptibility to TMEV (Campbell et al., 2001), and HSV-1 infection (DeLano, and, Mallery, 1998; Anglen et al., 2003).

To explore a possible connection between the immunosuppressive effects of RS and T-bet, we first demonstrated that RS reduced mRNA and protein levels of the

transcription factor T-bet isolated from primary splenocyte cultures. These stress effects were not entirely attributable to altered T-cell populations, as stress only moderately affected $CD4^+$ and $CD8^+$ T-cell percentages. The mechanisms by which stress results in a decrease in nuclear levels of T-bet is not currently understood, although several explanations exist. For instance, T-bet is known to be induced by IFN-γ dependent activation of STAT1 (Afkrian et al., 2002), a process that glucocorticoids have been shown to inhibit (Hu et al., 2002). Alternatively, it has been shown that the polarization of Th1 cells is dependent on the binding of IL-12(p70) to its receptor and the subsequent phosphorylation of STAT4 (Usui et al., 2006; Wurster et al., 2000). As mentioned previously, glucocorticoids can inhibit IL-12 production and secretion at the DC level which, in turn would conceivably reduce the number of T-bet positive cells. Supporting this hypothesis is the fact that RS caused significant decreases in both circulating serum IL-12(p40) and IL-12(p70) concentrations (Table 3-2). This process could also be ablated downstream of the signaling pathway, as dexamethasone has also been shown to inhibit the phosphorylation of STAT4 by a mechanism that was independent of IL-12R activation (Franchimont et al., 2000).

Additionally, we were able to demonstrate that DEX decreased virus peptide-specific IFN-γ secretion from splenocytes isolated from I/NR mice in a dose-dependent fashion. This effect did not appear to be entirely due to decreases in cell viability as determined by an MTT assay. As such, these results may indicate that the effects of DEX were acting on a molecular level rather than cellular. This effect was seen in both virus-specific $CD4^+$ T-cells and $CD8^+$ T-cells, directly indicating potential roles for

glucocorticoids in modulating the functions of either IL-12-induced STAT4, T-bet, Eomes or all of these factors at the *ifng* promoter. These possibilities are the subject of current investigation. However, it is likely that glucocorticoids exert their immunosuppressive properties concurrently at both the innate and adaptive levels of immunity.

Antibodies generated against Theiler's virus play an important role in viral clearance, as demonstrated by the fact that $CD4^+$ T-cell depleted mice can be "rescued" by passive transfer of anti-viral antibodies (Borrow et al., 1993). Immunization of SJL mice with the immunodominant antibody epitope ($VP1_{12-25}$) prior to infection with TMEV delays the onset of demyelination (Yahikozawa et al., 1997). In C57BL/6 mice antibodies are able to convey resistance in the absence of a $CD8^+$ T-cell response (Kang et al., 2005). Additionally, deletion of B-cells by administration of anti-IgM antibody to SJL mice during acute TMEV infection results in a more severe demyelinating disease (Rodriguez et al., 1990). Together, these studies illustrate the contribution of anti-TMEV antibodies in controlling early viral replication. Unlike virus-specific cellular responses, virus-specific humoral responses were found to be increased as a consequence of restraint stress, an unexpected finding given the nature of RS on virus-specific T-cell responses. Nonetheless, these effects were most readily illustrated by the increased virus specific Ig in the serum at days 8 and 16 p.i., although not producing statistically significant effects, the results from our virus-specific ELISPOT assays seemed to agree with this finding. Importantly however, no virus-specific antibody secreting cells were detected in the CNS of infected mice at either time point, a finding

that is supported by others (Pachner et al., 2007). This is in contrast to the results obtained for virus-specific $CD8^+$ T-cells which were abundantly present in the CNS of infected animals at both day 8 and 16 p.i. The mechanism by which stress affects the humoral response is not completely understood, and much conflicting evidence exists. For instance, our findings that stress results in increased virus-specific antibody levels are supported by the findings of (Brenner and Moynihan, 1997) demonstrating that in HSV-1 infection, uncontrollable footshock increased virus-specific serum IgM. Additionally, restraint stress also resulted in increased antigen specific IgM and IgG levels following immunization with KLH (Karp et al., 2000). However, our study is in contrast to studies conducted by others indicating that chronic stress decreases antigen specific antibody levels (Fukui et al., 1997; Houri-Haddad et al., 2003; Silberman et al., 2003), as well as with other studies that indicate stress has no effect on antibody levels (Sheridan et al., 1991; Feng et al., 1991; Karp et al., 1997; Cao et al., 2003). While we demonstrate significantly increased serum antibody levels, we acknowledge that the relevance of these findings remain obscured, as we have yet to measure the effects of stress on neutralizing levels of anti-virus antibodies.

There is little doubt about the occurrence of inflammatory processes within the CNS during the natural progression of MS. However, when considering that post mortem examination of newly developing lesions, indicate death of oligodendrocytes by apoptosis in the absence of inflammation (Barnett and Prineas, 2004), it seems pertinent to investigate the early event(s) leading to the manifestation of this disease. To this end, there is some evidence supporting the involvement of an infectious trigger in MS. For

instance, many chronic demyelinating diseases in animals are brought about by viral infection including canine distemper in dogs (Wisniewski et al., 1972); visna virus in Icelandic sheep (Sigurdsson et al., 1957) as well as MHV and Semliki Forest virus in mice (Weiner, 1973). In man the autoimmune demyelinating diseases transverse myelitis, acute disseminating encephalomyelitis and Guillan Barre syndrome, occur after infection or vaccination. Taken together, this indicates that overactive immune responses in response to viral infection or challenge can lead to demyelination and autoimmune-mediated myelin destruction.

Complicating matters, stress which results in the release of immunosuppressive steroids into the circulation, has been associated with both onset and exacerbation of the disease (Li et al., 2004; Grant et al., 1989; Mohr et al., 2004). If MS adheres strictly to an autoimmune pathogenesis then the effects of stress on the natural history of the disease are paradoxical. This is best illustrated by the effects of RS on the progression of EAE (Krakowski and Owens, 1996; Ferber et al., 1996; Willenborg et al., 1996). However if an infectious, perhaps a persistent agent, is involved in the initiation and progression of MS, then the immunosuppressive effects of stress, as occurs in Theiler's virus infection, will favor the pathogen and, after the resolution of the stressful stimuli, may expediate the autoimmune process, possibly through the attraction of naturally occurring autoreactive cells into the CNS from the periphery or by molecular mimicry and subsequent epitope spreading. The recent finding that bone marrow reconstitution in a chronic myelogenous leukemia patient with MS was unable to alter disease course may support the above concept (Jeffery, 2007).

Alternatively, it is known that viral infections, particularly upper respiratory infections, precipitate MS exacerbation (Buljevac et al., 2002). Through immunosuppressive properties, stress may increase the risk of MS exacerbation by indirectly increasing the risk of acquiring an infection. Supporting this hypothesis is the fact that experimental stress in humans followed by rhinovirus infection resulted in increased disease severity (Cohen et al., 1991). Nevertheless, our results indicate that stress results in immunosuppression of both TMEV specific T-cell responses, particularly protective IFN-γ, and provides a sound mechanism for observed increased CNS viral titers and dissemination during early Theiler's virus infection (Mi et al., 2006a).

IV RESTRAINT STRESS FAILS TO RENDER C57BL/6 MICE SUSCEPTIBLE TO THEILER'S VIRUS INDUCED DEMYELINATION

1. Introduction

Multiple sclerosis (MS) is a chronic idiopathic demyelinating and neurodegenerative disease of the central nervous system. As such, both the onset and exacerbation of MS are currently thought to be heavily influenced by multiple factors including infectious, genetic and environmental (Hauser and Oksenberg, 2006). Evidence suggesting a viral involvement in the etiology of MS includes the association of multiple viral infections with MS (Cermelli and Jacobson, 2000), the precedence of relapses by infection (Sibley et al., 1985; Panitch, 1994) and epidemiological studies demonstrating MS outbreaks in areas with no previous history of the disease, particularity those conducted on the Faroe Islands (Kurtzke and Heltberg, 2001). Additionally, human viral infections are known to cause demyelination both through direct lysis of oligodendrocytes, as occurs in progressive multifocal leukoencephalopathy brought on by JC virus infection, and indirectly**,** as is the case in post infectious acute disseminated encephalomyelitis initiated by vaccination or infection (Tenembaum et al., 2007) and subacute sclerosing panencephalitis caused by measles virus infection (Sips et al., 2007). In addition, virus-induced demyelination is demonstratable in animals as well. For instance, infection of Icelandic sheep with visna virus (Sigurdsson et al., 1957), dogs with canine distemper virus (Wisniewski et al., 1972), and mice with Semliki forest virus, the JHM strain of murine hepatitis virus, or

Theiler's murine encephalomyelitis virus (Weiner, 1973; Daniels et al., 1952; Lipton, 1975) all induce demyelinating diseases of the CNS that pathologically resemble human MS.

Aside from evidence supporting an infectious trigger for MS onset and exacerbation, genetic predisposition to the disease is also likely to play a role in the pathogenesis of MS. This is illustrated by studies conducted on twins that demonstrate an approximate 30% concordance rate among monozygotic twins but only a 3-5% rate among dizygotic twins (Ebers et al., 1986; Willer et al., 2003; Hansen et al., 2005a; Hansen et al., 2005b). Indeed, multiple loci representing nearly every chromosome have been associated with MS (Dyment et al., 2004). However, to date, the strongest and most reproducible genetic link, accounting for an estimated 17-60% of the genetic association with MS, is the HLA gene HLA-DRB1 (Haines et al., 1998; Hauser and Oksenberg, 2006).

Other environmental stimuli, such as psychological stress, have also been implicated in the involvement of MS. Interestingly, stress has been repeatedly associated with both onset (Warren et al., 1982; Grant et al., 1989; Li et al., 2004) and exacerbation (Mohr et al., 2004; Brown et al., 2006a; Brown et al., 2006b) of the disease, although the mechanism(s) behind these effects of stress on MS are currently poorly understood.

Theiler's murine encephalomyelitis virus (TMEV) is a member of the family *Picornaviridae*. TMEV infection of mice provides an excellent model in which to study human MS because it is endemic in mice although, in nature, rarely causes

demyelinating disease (Theiler, 1934). Moreover, the development of Theiler's virus induced demyelinating disease (TVIDD), the chronic progressive immune-mediated demyelinating and neurodegenerative disease, has been shown to depend on viral persistence within the CNS. This persistence is genetically influenced by multiple loci both within and outside the murine H-2 (Brahic et al., 2005). As such, mice that are H-2 ^{d,b,k} are able to effectively clear the virus from the CNS and do not develop TVIDD, mice that are H-2 ^{s,v,q,r,f} do not clear the virus from the CNS and eventually develop TVIDD, and congenic strains such as B10.S demonstrate reduced severity of TVIDD (Azoulay-Cayla et al., 2000; Rodriguez et al., 1986)

It is for these reasons that we have been interested in the mechanisms by which stress can influence the pathogenesis of TMEV infection. We have previously found that psychological stress administered during early infection of TMEV in susceptible SJL mice results in an earlier and exacerbated demyelinating disease (Sieve et al., 2004). We hypothesized that immunosuppression during acute infection, resulted in higher viral titers in the CNS, which in turn, contributed to earlier onset and exacerbation of TVIDD. Because adaptive immunity, specifically virus specific CD8 T cells responses, are required for viral clearance from the CNS of non-susceptible C57BL/6 mice (Mendez-Fernandez et al., 2003), we questioned whether chronic stress during early infection would alter susceptibility of the normally resistant C57BL/6 strain to TVIDD.

2. Methods and materials

2.1 Mice

Female C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine). The animals were housed 3-4 per cage, and kept on a constant 12 hour light-dark cycle (0500/1700 h). Food and water was available to all mice *ad libitum*. Upon arrival mice were equally distributed into cages based on body mass. Before the onset of each experiment, all mice were divided into groups corresponding to infection and stress, allowed to acclimate for 1 week prior to the onset of the experiment. To determine the effects of RS on infection during the acute infection with TMEV infected non-restained mice (I/NR) were compared to infected restrained mice (I/R). To test the effects of stress on susceptibility to chronic demyelination I/NR, I/R and sham infected restrained (NI/R) mice groups were used.

2.2 Virus and infection

All mice were anesthetized with isoflourane prior to the infection. Mice in the infected groups were then injected intracerebrally (i.c.) with approximately 5.0×10^5 p.f.u. of the BeAn strain of TMEV (kindly provided by Dr Lipton), and mice in the non-infected groups were sham infected (i.c.) with sterile phosphate buffered saline.

2.3 Restraint stress procedure

Restraint stress was carried out as described previously (Mi et al., 2006a). Briefly, mice in the restraint group were restrained starting 1 day prior to infection for 8

hours a session (1200-0800). For acute phase experiments, mice were restrained nightly for 7 consecutive days. For chronic phase experiments, mice received a total of 20 restraint sessions consisting of 5 consecutive nights of stress followed by 2 nights with no stress (Sieve et al., 2004).

2.4 Physiological indices of stress and infection

The degree of ruffling, an indicator of stress, as well as body mass were recorded daily during the acute phase experiments (day 7 or 16 p.i.), and biweekly during the chronic phase experiments. The degree of ruffling was scored as 0 = no ruffling, 1 = ruffling of the head, 2 = ruffling of the head, and 50% of the body, 3 = ruffling of the head and entire body.

2.5 Blood collection for RIA

Prior to restraint, and 48 hours post-restraint, all mice were bled from the saphaneous vein as previously described during the hours of 0800 and 1000 (Sieve et al., 2004). Immediately following collection, plasma was removed after centrifuging at 3000 x g for 10 minutes at 4°C, and stored at -80˚C until corticosterone levels could be determined by radioimmunoassay (RIA; Keith et al., 1978).

2.6 Corticosterone assay

All plasma were assayed for corticosterone at baseline (before restraint) and 48 hours post restraint using RIA kits obtained from MP biomedicals (Morgan Irvine, CA)

according to the manufacturers' instructions (Keith et al., 1978). Corticosterone levels are expressed in ng/ml.

2.7 Measurement of serum amyloid a levels

Serum Amyloid A (SAA) levels were measured from serum by ELISA according to manufacturers' instructions (Biosource).

2.8 NK cell assay

NK cell activity was assessed using a standard ^{51}Cr release assay as previously described using splenocytes as effectors and YAC cells as targets (Welsh et al., 2004). Total ^{51}Cr release was determined by incubating targets with 10% Triton-X 100. Spontaneous release was determined by counting the amount of ^{51}Cr released after incubation of targets in complete RPMI-1640 media. Total lysis of experimental samples were calculated using the following equation: [(experimental release – spontaneous release)/(total release – spontaneous release)] x 100. All samples were run in triplicate.

2.9 Antibody levels

TMEV-specific antibody levels from serum of mice taken from days 7, 16, 73, 155, and 193 p.i. were determined using ELISAs developed in our lab as described previously (see above).

2.10 CTL assay

Cytotoxic T lymphocyte activity was assessed on days 7 and 16 using a chromium release assay. Here, previously isolated syngeneic cerebrovascular endothelial (CVE) cells (Sapatino et al., 1993) were infected with the BeAn strain of TMEV at a multiplicity of infection (m.o.i.) of 1 for 24 hours. The cells were then radiolabelled with 100μCi/2.0 x 10^6 cells with $Na^{51}CrO$ for 2 hours at 37° C and 5.0% CO_2. Meanwhile, single cell suspensions of spleen cells were made as described previously (Welsh et al., 2004), and added to sterile round-bottomed 96-well polystyrene plates at a concentration of 1.0 x 10^6 cells/well in a total volume of 100μl media. Doubling dilutions were made of effectors, and 1.0 x 10^4 radiolabelled CVE cells per 100μl media were added to all wells. The cells were then brought together by centrifugation at 500 x g for 1 minute. The plate was incubated at 37° C and 5.0% CO_2 for 4 hours. The cells were pelleted by centrifugation at 2000 x g for 20 minutes, and 100 μl of the supernatant was removed and counted using a gamma counter. Total ^{51}Cr release was determined by adding 100 μl of 10% Triton-X to wells containing radiolabelled CVE cells only. Spontaneous release was determined by measuring 100 μl of supernatant containing only radiolabelled CVE cells. Percent lysis was calculated using the following formula: [(experimental release – spontaneous release)/(total release – spontaneous release)] x 100. All samples were run in triplicate.

2.11 Plaque assays

Plaque assays were used to determine viral levels in the brains of mice at days 7 and 16 post infection as described previously (Mi et al., 2006b).

2.12 Rotarod analysis

Rotarod analysis can serve as an indicator of motor dysfunction as a result of Theiler's virus induced demyelinating disease, if measured during the chronic phase of TMEV infection (McGavern, et a., 1999). Therefore, all groups were tested for performance on rotarod starting at day 77 p.i. using a Ugo Basile Model 7650 accelerating rotarod treadmill. The time on the rod comprised 1 session and was recorded for each animal. Each animal completed two sessions on each test date. Scores were taken biweekly, and results were expressed as the means of two sessions per date.

2.13 Histology

Mice in the chronic phase experiment were sacrificed at day 195 p.i., and perfused with 10% formalin. The brains and spinal cords were isolated, grossly sectioned, dehydrated, embedded in paraffin, sectioned into 5μm sections and stained with hematoxylin and eosin (H+E). Sections were then examined for inflammation and demyelination.

2.14 Statistics

Data are presented as mean ± SEM. Data were analyzed using analysis of variance (ANOVA) and repeated measures ANOVA. When appropriate, Fisher's protected least significant difference model was used for post hoc comparison of means. In all cases significance was set at $\underline{p} \leq 0.05$.

3. Results

3.1 Experiment 1: Early infection

3.1.1 Indices of stress

Mice that have undergone restraint stress typically display ruffled fur and decreased body weight (Sieve et al., 2004). As seen in Figure 4-1, stress caused a significant decrease in body weight which corresponded to the period in which mice were receiving nightly restraint sessions $\underline{p} < 0.01$(days 1-7 p.i.) (Fig. 4-1A). This effect was immediately diminished following the termination of these sessions and no differences between I/NR vs I/R mice were seen for the duration of the experiment (days 8-16 p.i.) $\underline{p} = 0.815$ (Fig. 4-1A). In contrast, mice in the I/R group displayed significantly increased ruffling scores when compared to I/NR mice (Fig. 4-1B). This effect was more significant at days 1-7 p.i., corresponding to the time in which I/R mice were being stressed $\underline{p} < 0.0001$. However, I/R mice remained more ruffled than I/NR mice even after stress sessions had ceased (days 8-16 p.i.) $\underline{p} < 0.01$ (Fig. 4-1B). These results clearly demonstrate differences in groups based on stress.

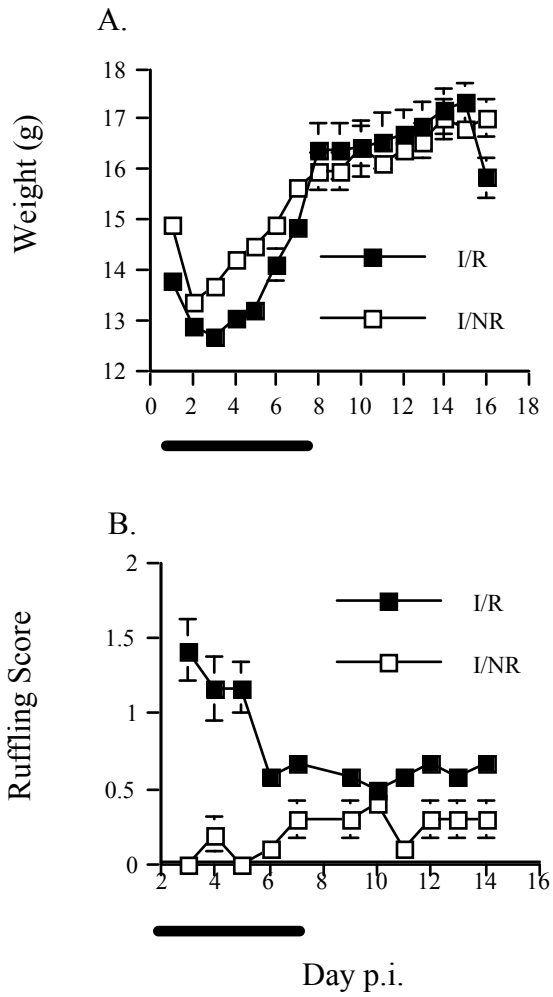


Figure 4-1. Restraint stress decreases body weight and causes ruffling in TMEV-infected C57BL/6 mice. During the restraint stress (RS) period (solid black line), infected/restrained (I/R) mice showed significant weight loss compared to infected/non-restrained (I/NR) mice ($\underline{p} < 0.01$) (A). RS significantly increase ruffling scores in I/R vs. I/NR mice during the restraint period ($\underline{p} < 0.0001$) (B). These effects were quickly diminished after the RS sessions ceased (A-B).

3.1.2 Innate immune function

Natural killer (NK) cells play an important role in the early limitation of viral infection through both the direct lysis of viral infected cells as well as the secretion of IFN-γ. In C57BL/10 mice, the depletion of NK cells by antibody during early infection with Theiler's virus resulted in increased gray matter disease (Paya et al., 1989). As we have demonstrated that RS decreases NK cell activity in susceptible CBA mice (Welsh et al., 2004), we questioned if stress could also decrease the NK cell response in C57BL/6 mice. Previous pilot experiments indicated day 1 p.i. as having the most significant virus-specific increases in NK cell lysis (not shown). Therefore, this time-point was used to determine the effects of stress on NK cell effector function. As seen in Figure 4-2, stressed mice displayed significantly decreased overall NK cell responses when compared to their non-stressed counterparts, and infected mice had higher overall NK cell responses compared to non-infected mice (Fig. 4-2A; $\underline{p}s < 0.001$). We found that the effect of stress was most significant at the effector to target ratio of 100:1 while not statistically significant, NK cell lysis of YAC-1 target cells appeared to be modestly decreased from 36% in I/NR mice to approximately 25% in I/R mice accounting for a reduction of about 11% ($\underline{p} = 0.068$; Fig. 4-2B). This result may demonstrate a stress induced decrease in innate immune processes which has shown to protect against gray matter disease (Paya et al., 1989).

Alternatively, at day 1 p.i., both infection with TMEV and stress were found to significantly increase circulating levels of the acute phase reactant serum amyloid A, a protein typically used as a marker of innate inflammation following viral and bacterial

infections (Fig. 4-2C; both p's > 0.001). Together these results indicate that only selective aspects of innate immunity are reduced by chronic stress, and that stress may actually facilitate the initiation of some inflammatory responses.

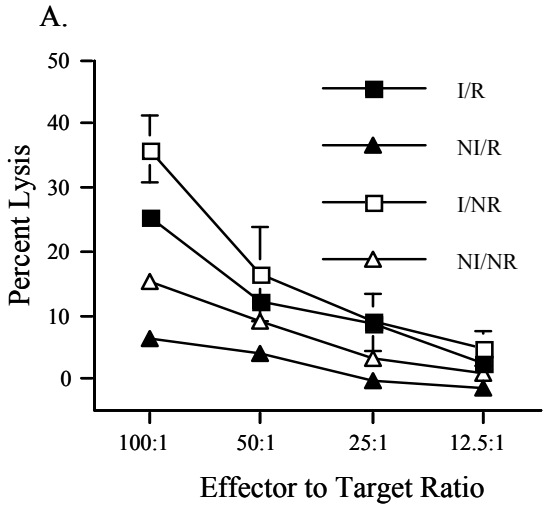


Figure 4-2. Restraint stress differentially affects markers of innate immune function at day 1 p.i. Mice were stressed by restraint one day prior to infection with TMEV. At day 1 p.i. splenic NK cell effector function was measured by chromium release assay using YAC-1 cells as targets. At an effector:target ratio of 100:1 (B) I/R mice (n = 5) displayed a strong trend towards reduced capacity to lyse targets than I/NR mice (n = 4; p = 0.068). NI/R mice (n = 4) had significantly lower NK cell activity than NI/NR mice (n = 4; p < 0.001). Serum SAA levels from mice at day 1p.i. were tested by ELISA (C) and indicated that both infection and stress drastically increased SAA levels (p's < 0.001). Shown are the means ± S.E.M.

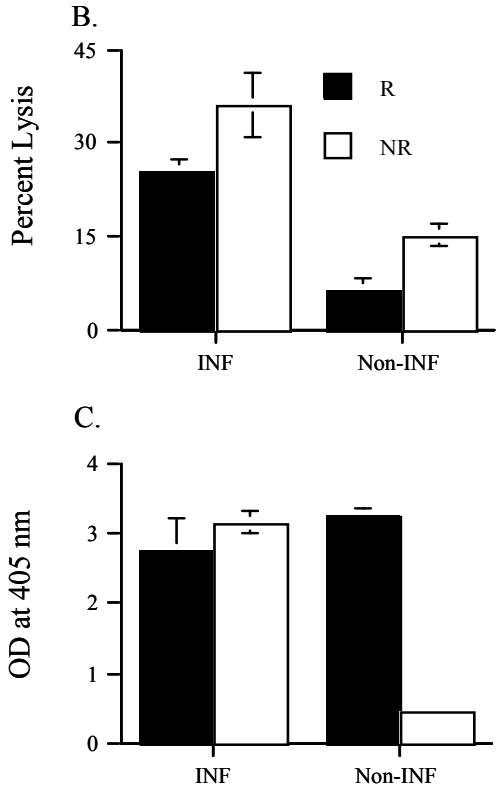


Figure 4-2, continued.

3.1.3 Effects of RS on adaptive immune responses to TMEV at days 7 and 16 p.i.

The resistance of C57BL/6 mice to Theiler's virus induced demyelination is due to their ability to effectively clear TMEV from the CNS during early infection. In fact, practically all virus is eliminated from these mice by day 16 p.i. after i.c. inoculation (Dethlefs et al., 1997). Early viral clearance in C57BL/6 mice has been attributed to the generation of a robust adaptive immune response, which utilizes both cellular and humoral immunity (Kang et al., 2005). We determined the effects of RS on these responses at days 7 and 16 p.i. Interestingly, at day 7 p.i. no differences were detected

between groups although it appeared that I/R mice had reduced spleen mass (106 ± 23 mg) compared to I/NR mice (158 ± 14 mg), but this effect was not statistically significant $\underline{p} = 0.095$ (Fig. 4-3A). Likewise, at day 16 p.i, no differences between I/R and I/NR groups were found to exist (Fig. 4-3A). This was in sharp contrast to the effects of stress on CBA mice (Welsh et al., 2004).

The cytotoxic T-lymphocyte (CTL) response plays a major role in TMEV clearance and resistance to demyelination in C57BL/6 mice (Mendez-Fernandez et al., 2003). In these mice, the CTL response is detectable as early as 5 days post infection, peaks between days 7-10 p.i. and resolves with viral clearance by day 16 p.i. (Dethlefs et al., 1997a). Using infected cloned syngeneic CVE cells as targets in a chromium release assay, we observed no differences between I/NR and I/R groups (Fig. 4-3B; $\underline{p} = 0.2588$) at day 7 p.i.. By day 16 p.i., the CTL response had resolved and was undetectable in I/NR and I/R mice groups (Fig. 4-3C).

Antibody responses directed toward TMEV have also been shown to limit viral persistence in the CNS in the absence of a $CD8^+$ T-cell response, illustrating their importance in viral neutralization during early infection and protection from encephalitis (Kang et al., 2005). Therefore, we measured the effects of stress on virus-specific IgM and IgG responses by ELISA at both day 7 and day 8 p.i. We found no stress-induced differences in IgM responses at either day 7 or day 16 p.i. both (both $\underline{p}s > 0.05$). Additionally, the virus-specific IgM response was not found to differ between day 7 and 16 p.i. (Fig. 4-3D-E; $\underline{p} > 0.05$). In contrast to the IgM response, the virus specific IgG was almost undetectable at day 7 p.i., but was substantially increased by day 16 p.i. (Fig.

4-3F-G; $\underline{p} < 0.0001$). However, RS was not found to significantly influence the level of circulating virus-specific IgG at either time-point (Fig. 4-3F-G; $\underline{p} > 0.05$). Together these results indicate that RS did not significantly alter virus-specific immune responses at either day 7 or 16 p.i.

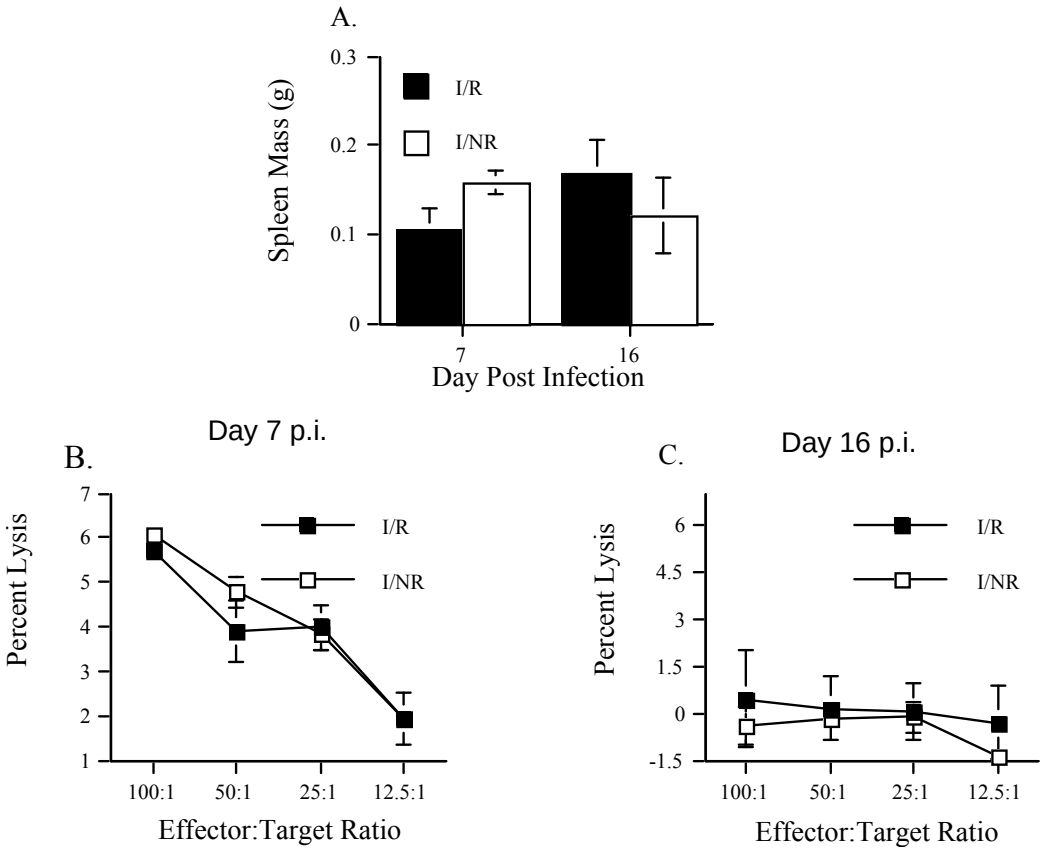


Figure 4-3. Effects of RS on adaptive immune responses to TMEV at days 7 and 16 p.i. A. Spleen weight at day 7 p.i. and at day 16 p.i. B-C. Splenic CTL responses against infected CVE cells at day 7 p.i. (B) and at day 16 p.i. (C). D-E. Anti-TMEV IgM levels from serum at day 7 (D) and day 16 p.i. (E). F-G. Serum IgG levels to TMEV, at day 7 (F), and day 16 (G). Results are means ± S.E.M.

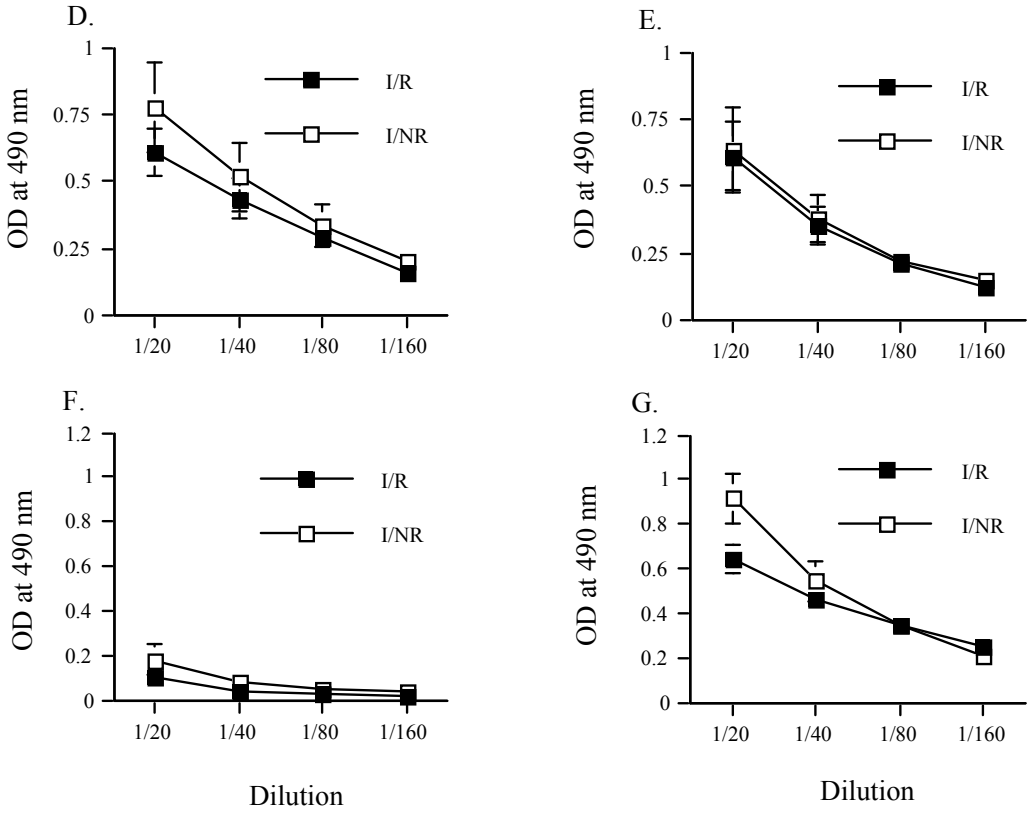


Figure 4-3, continued.

3.1.4 Effects of RS on brain viral titers

In order to determine the effects of RS on viral persistence within the CNS, we used plaque assays to measure viral titers from brains at days 7 and 16 p.i. Figure 4-4 illustrates the effect of RS on brain viral titers. Collectively, viral titers were highest on day 7 p.i. compared to day 16 p.i. (Fig. 4-4). It appeared that I/R mice had slightly higher viral titers than I/NR mice at day 7 p.i., but this finding was not statistically significant ($p > 0.05$). The same was true at day 16 p.i., insofar as there were no

significant differences between I/R vs. I/NR groups (Fig. 4-4). These results indicate that RS did not substantially alter the ability of mice to clear virus from the CNS.

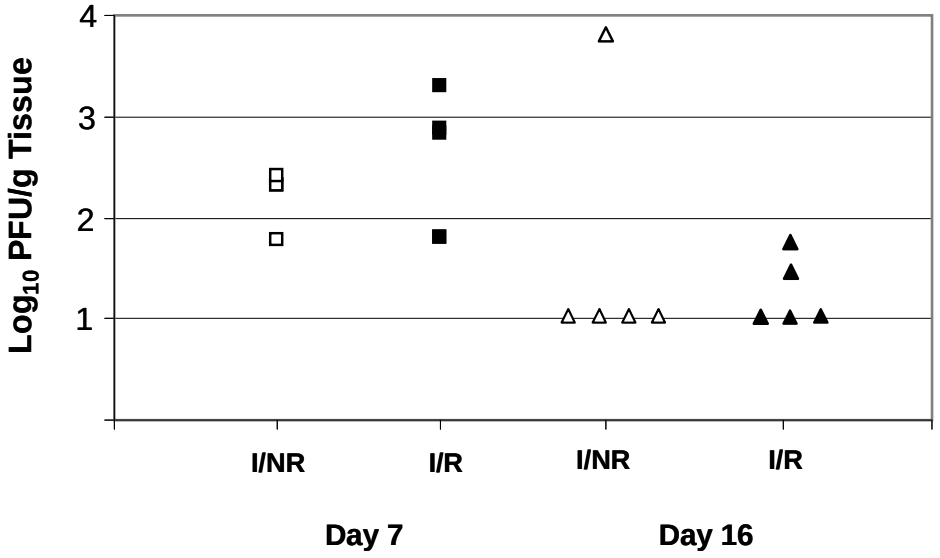


Figure 4-4. Effects of RS on brain viral titers at day 7 and 16 p.i. Brain viral titers were measured by plaque assay on days 7 and 16 p.i. I/R mice had slightly higher levels of virus at day 7 p.i. (left) than I/NR, but the effect was not significant. By day 16 p.i. (right) both I/R and I/NR mice had low or undetectable levels of virus in the brain, and no effects of RS were noted.

3.2 Experiment 2: Late infection

Results from our first experiment indicated that 8 consecutive RS sessions each lasting 8 hours did not significantly alter either virus specific adaptive immunity or viral persistence in the early phases of TMEV infection. Due to the age of our mice (3-5 weeks old) we could not increase the length of each stress session without expecting high mortality rates (Campbell et al., 2001). Therefore, we altered the number of RS

sessions such that mice received 5 consecutive nights of RS with 2 night rest in between, for a total of 15 RS sessions rather than 8 (Sieve et al., 2004). To ensure that 8 hours of RS was acting as a stressor to our C57BL/6 mice, we measured serum corticosterone before and immediately after the second RS session as an indicator of hypothalamic-pituitary-adrenal (HPA) axis activation (Sieve et al., 2004). C57BL/6 mice do not develop demyelination following infection with TMEV. However, in order to determine the effects of RS on altered susceptibility to TMEV, we included NI/R mice as a control group.

3.2.1 Effects of stress on body weights, ruffling, and plasma corticosterone concentration

As in experiment 1, we found that RS significantly decreased body weights of mice in the restrained groups (I/R and NI/R) during the period in which the mice were receiving nightly RS sessions (days 0-21p.i.) (Fig. 4-5A; $\underline{p} < 0.0001$). Body weights were found to quickly returned to levels comparable of I/NR mice immediately following RS sessions, and were not found to differ again for the duration of the experiment (days 22-193) (Fig. 4-5A; $\underline{p} > 0.05$). However, infection with TMEV had no effect on body weights at any time-point $\underline{p}$'s > 0.1 (Fig. 4-5A).

Also, we found that ruffling scores were significantly increased during days 0-21 p.i. in both I/R as well as NI/R groups compared to the I/NR group (Fig. 4-5B; $\underline{p} < 0.0001$). These scores remained significantly increased following the restraint period

(days 22-193 p.i.) for mice in the restrained groups (Fig. 4-5B; $\underline{p} < 0.0001$). Once again, infection did not alter signs of ruffling at any time-point (Fig. 4-5B; $\underline{p}$'s < 0.05).

As illustrated in Figure 4-5C, we found that restraint stress significantly increased plasma corticosterone in I/R, and NI/R mice compared to I/NR mice ($\underline{p} < 0.0001$), while infection had no influence on plasma corticosterone (Fig. 4-5C; $\underline{p} > 0.05$) demonstrating HPA axis activation following stress. Taken together, the above data indicate that mice receiving restraint stress were indeed stressed.

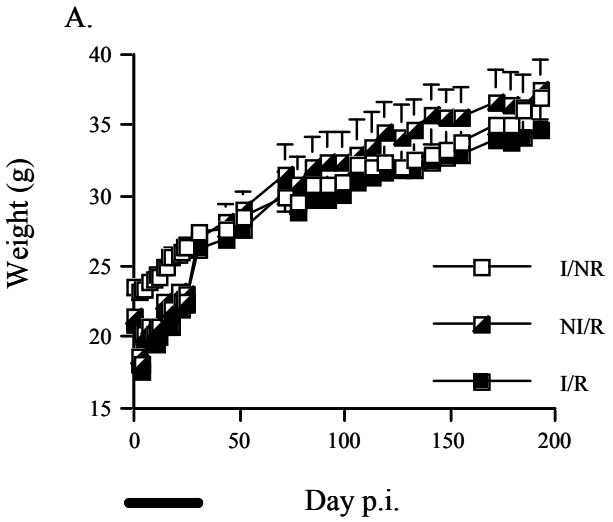


Figure 4-5. Effects of infection and RS on weight, ruffling and plasma corticosterone. A-B. As in the acute phase study, restrained mice displayed decreased body weight (A.) and ruffling scores (B) compared to non-restrained mice during the RS period (solid black line) ($\underline{p} < 0.001$), but this effect quickly subsided following the RS period (A-B). C. Plasma CORT levels were not different between groups prior to restraint, but RS increased CORT at 48 hours after the first restraint session (day 1 p.i.) ($\underline{p} < 0.0001$) (C). Results are means ± S.E.M. $*P < 0.05$, $***P < 0.001$ respective to pre-restraint controls.

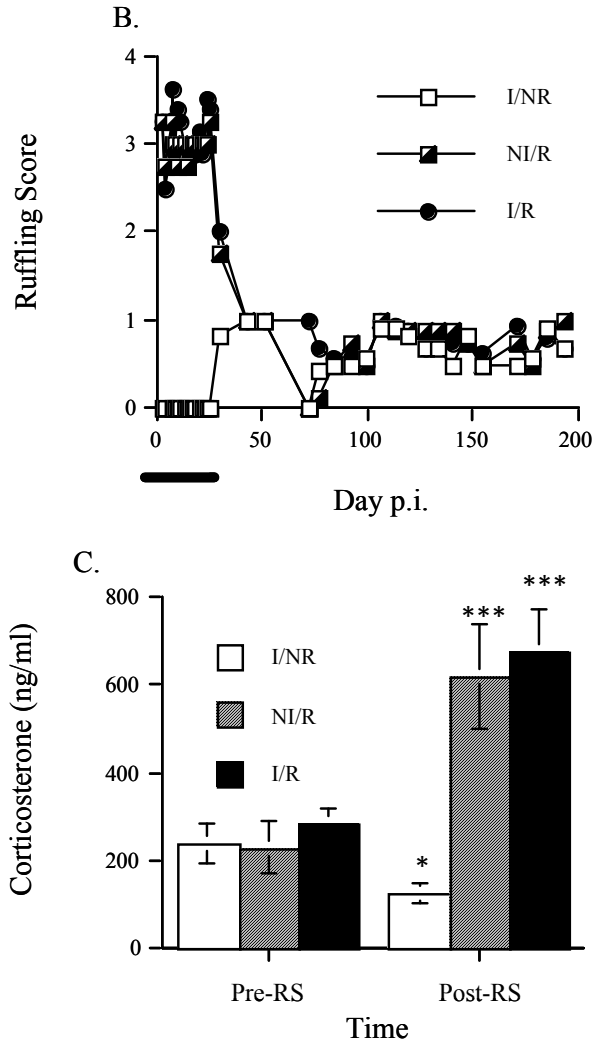


Figure 4-5, continued.

3.2.2 Effects of stress on TMEV-specific antibody levels at days 74, 127, and 193 p.i.

Virus-specific IgG antibody levels were used as indicators of the effects of chronic stress occurring during early disease on adaptive immunity during the late phase of TMEV infection. Therefore, virus-specific circulating antibody levels were determined by ELISA. Infection with TMEV resulted in substantially increased antibody levels at days 74, 127 and 193 p.i. when compared to non-infected mice groups (I/NR and I/R vs. NI/R; Fig. 4-6A-C). However, as occurred during early infection (days 7 and 16 p.i.; Fig. 4-3F-G) previous restraint stress was not found to alter the humoral response, as measured by serum IgG levels to TMEV at any of these time-points post infection (Fig. 4-6A-C). These results indicate that despite 15 sessions of restraint stress occurring during early infection with TMEV the mice in the I/R group were still able to mount an effective, and robust antibody mediated immune response toward TMEV.

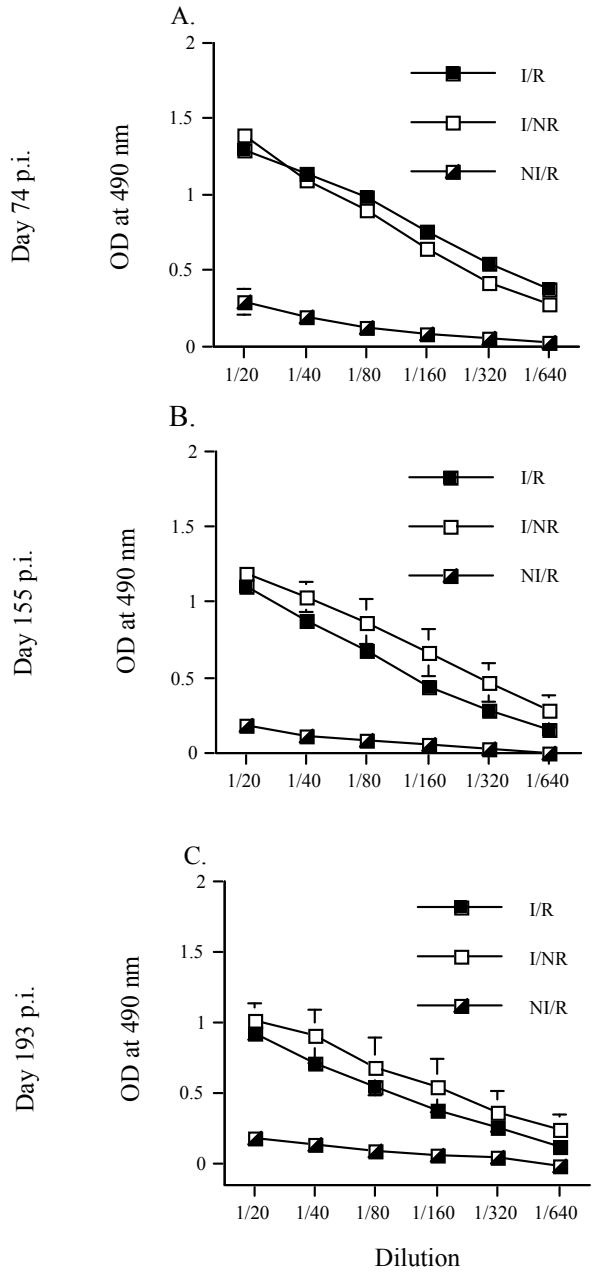


Figure 4-6. Effects of RS on serum virus-specific IgG responses to TMEV at days 74, 155, and 193 p.i. Infected mice had higher antibody levels than non-infected controls at days 74 (A), 155 (B) and 195 (C) p.i. RS had no effect on antibody levels at these time points (A-C). Results are means ± S.E.M.

3.2.3 Effects of stress on indices of demyelination

Susceptible mice infected with TMEV, such as the SJL strain, display clinical symptoms of demyelination which include a wobbly gait, abnormal righting reflex, a hunched posture, and ruffling of the fur. The onset of these symptoms typically begins around 40-55 days post-infection and progressively worsens as the animal ages. However, as hunched posture and ruffled fur can also be used to assess degree of stress (Figs. 4-1B and 4-5B), we focused primarily on gait score as an indication of disease onset. Additionally, we tested motor responses by rotarod bi-weekly. No mice developed signs of gait abnormality at any time during the experiment (Not shown). Moreover, as seen in Figure 4-7A, neither infection nor restraint stress altered the degree of motor capability as determined by rotarod analysis (both $ps > .05$). Finally, histological analysis of spinal cords from I/R mice demonstrated no inflammation or demyelination (Fig. 4-7B).

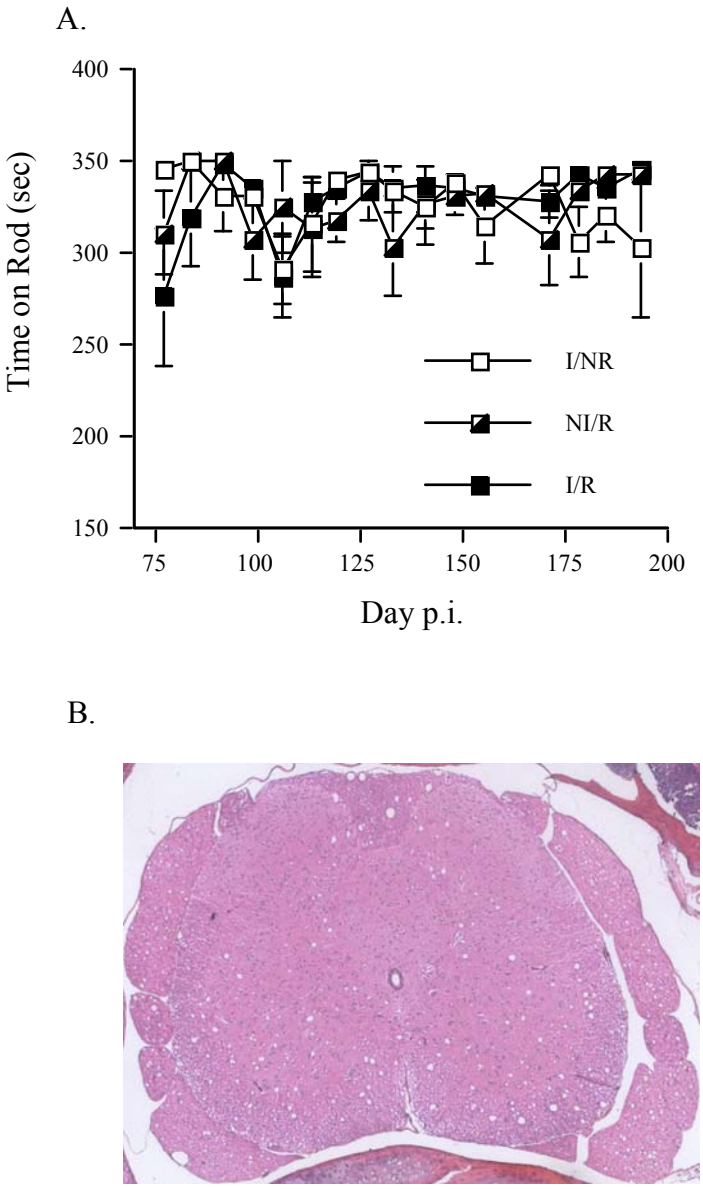


Figure 4-7. Effects of RS on indices of demyelination. No differences were detected between I/R, I/NR or NI/R groups by rotarod (A). H+E staining of CNS tissue isolated at day 193 p.i. demonstrated no inflammation or demyelination in I/R mice (B).

4. Discussion

Restraint stress immediately preceding and following influenza infection has been reported to decrease NK cell activity (Tseng et al., 2005; Hunzeker et al., 2004), decrease CTL responses (Sheridan et al., 1998) mononuclear cell infiltrates into the lungs (Hermann et al., 1995) and decrease IL-2 production in the lungs (Sheridan et al., 1991). Likewise, RS has also been shown to downregulate CTL, NK and IL-2 responses to experimental herpes virus infection (Bonneau et al., 1991), leading to increases in HSV induced encephalitis (Anglen et al., 2003), as well as cause viral reactivation from the trigeminal nerves of infected mice (Freeman et al., 2007).

During early infection with TMEV, 12 hour sessions of RS in CBA mice has been shown to decrease NK cell activity (Welsh et al., 2004), chemokine, and cytokine expression in the brains and spleens, as well as alter viral dissemination and viral load (Mi et al., 2004; 2006a; 2006b). Additionally, 8 hour sessions of RS in SJL mice causes suppression of virus specific T-cell responses, in the CNS as well as the periphery (Steelman et al., unpublished observation) which may result in an exacerbated late disease (Sieve et al., 2004).

Based on our previous findings, as well as those of others, we hypothesized that 12 hour RS sessions would cause suppression of innate and adaptive immune responses toward TMEV in C57BL/6 mice – responses that are needed for viral clearance and inhibition of viral persistence from the CNS in this prototypical TMEV resistant inbred strain (Brahic et al., 1981; Rodriguez et al., 1995). Therefore, as occurs in susceptible strains of mice, we hypothesized that this immunosuppression would result in increased

viral load in the CNS, possibly leading to persistence and the subsequent development of TVIDD. Our results overwhelmingly demonstrate that 12 hours RS sessions of C57BL/6 mice do not alter susceptibility to TMEV, and failed to significantly suppress the development of both antibody and CTL responses directed toward TMEV (Fig. 4-3). These findings mirror the inability of RS to significantly influence viral load within the CNS of our infected mice (Fig. 4-4). While these data are in agreement with studies conducted on the effects of repeated 16 hour sessions of RS on virus specific humoral responses to influenza (Sheridan et al., 1991; Feng et al., 1991), they are in contrast to the findings of others that clearly demonstrate suppressive effects of repeated RS on CTL effector function (Bonneau et al., 1991; Sheridan et al., 1998; Freeman et al., 2007). One reason for the discrepancy between our data and those of others may be attributable to the subtle differences in the assays used to test the CTL response. While others have successfully used radiolabelled splenocytes as target cells, we chose to employ the use of cloned cerebrovascular endothelial cells, a suspected target of *in vivo* infection of TMEV and possibly persistence (Sapatino et al., 1995). While the degree of our percent lysis are low compared to conventional assays we believe that they accurately reflect the events occurring *in vivo* as illustrated which is illustrated by the fact that CNS viral load did not significantly differ between I/NR and I/R groups during the same timeframe. On the other hand, despite obvious differences in body weight and the increases in ruffling scores due to RS, we have also entertained the possibility that our mice were not stressed. However, RS clearly resulted in the release of corticosterone into the blood (Figure 4-5C) which is an accepted marker of HPA axis

activation. Moreover, these concentrations have been shown to correlate with immunosuppression in other strains of mice (Sieve et al., 2004).

Alternatively, this paradox may be explained in several ways. First, while our mice were certainly stressed, their allostatic load may not have been increased enough to cause immunologic distress (McEwen, 2007). This concept is supported by findings of others that demonstrate how the timing and duration of a stressor as well as the influence of the genetic background on the immune response (Harizi et al., 2007). As mentioned previously, C57BL/6 mice are capable of generating a very effective early immune response toward TMEV (Oleszak et al., 2004). Therefore, it could be possible that the antigenic stimulus alters the allostatic set point for when stress becomes distress. In this scenario, an equivalent amount of stress is likely to differentially affect the immunologic outcome of any given pathogenic stimuli based on its degree of immunogenicity. This could explain why the same dose of stress (12 hours) differentially affects viral titers in inbred mouse strains of varying susceptibility to TMEV (Mi et al., 2004; present data).

The onset of mulitple sclerosis is thought to be influenced by multiple factors (Hauser and Oksenberg, 2006). Of these, psychological stress has been repeatedly associated with MS since around the time of its initial characterization by Charcot (Charcot, 1877). However, while most studies support a role of stress in the pathogenesis of MS, some studies have demonstrated a lack of association between stressful life events and exacerbation of MS (Mohr et al., 2004). Using TMEV as a model for human MS, we have now demonstrated the ability of C57BL/6 mice to prevent persistent viral infection of the CNS despite being subjected to a chronic stressor

during a critical time in the determination of viral persistence (Oleszack et al., 2004). As such, this study may have important implications when considering the effects of stress on human diseases such as MS. For instance, different populations may vary in response to stress or have different allostatic set points, such that the perception of a similar stressor may not necessarily trigger a uniform distress in different individuals. This could explain why chronic stressors in certain populations are strongly linked to MS onset (Li et al., 2004) and exacerbations (Grant et al., 1989), whereas, other chronic stressors occurring in different populations, are not linked to MS exacerbations (Nisipeanu and Korczyn, 1993).

In conclusion, RS did not alter susceptibility to TMEV-induced demyelination of C57BL/6 mice. However, the data herein begin to elucidate a role for how genetic, immunologic and environmental factors contribute to virus-induced demyelination, in a sense validating the use of RS and TMEV infection as a model of human MS. At the very least, this study emphasizes the importance of using different inbred strains of mice to address the role of stress on host pathogen relationships.

V DISCUSSION AND CONCLUSIONS

1. General summary

The results of the studies herein demonstrate the effects of stress-induced immunosuppressive responses directed toward TMEV. Specifically, we were able to demonstrate early suppression of infection-induced splenic NK cell-mediated cytotoxicity which was paralleled by increased CNS viral titers in both CBA and BALB/c mice. In SJL mice, which are highly susceptible to TMEV-IDD, we were able to demonstrate alterations in virus-specific adaptive immune responses at a time in which viral clearance for this disease is paramount. In this regard, we found that the numbers of virus specific IFN-γ producing $CD4^+$ and $CD8^+$ T-cells were decreased in the spleens, and in the case of virus specific $CD8^+$ T-cells, the CNS. Importantly, this suppression by restraint stress was not restricted to type 1 immune responses, but also extended to type 2 responses as illustrated by reduced IL-10 and IL-4, and in the CNS, IL-17 responses as indicated by responses to UV-inactivated whole virus. By treating mice with dexamethasone alone or RU486 administered concurrently during stress, we were able to demonstrate a partial role for glucocorticoids in the inhibition of virus specific type 1 immunity. Finally, we showed that in TMEV-infected non-stressed SJL mice, virus-specific IFN-γ production was inhibited in a dose dependent fashion by co-culture with dexamethasone. In spite of the immunosuppressive effects on virus-specific T-cell responses, we found that restraint stress increased anti-viral serum Ig levels. Finally, we demonstrated that restraint stress does not have the same immuno-modulatory actions on C57BL/6 mice, as immune responses in these mice were hardly

affected by restraint stress. As a result, CNS viral clearance was not significantly delayed in this mouse strain, and stress did not overcome genetic predisposition to resistance. Taken together, these results support the conclusions of other studies that were suggestive of restraint-stress induced suppression of T-cell effector function, (Mi et al., 2004; 2006b), and are strongly suggestive of a mechanism for the observed increases in viral titers and dissemination (Mi et al., 2006a).

2. RS suppresses NK cell responses in both susceptible and resistant strains of mice

We demonstrated that two sessions of RS were sufficient to cause overall reductions in NK cell cytotoxicity and result in increased in CNS viral titers in both CBA and BALB/c mice. Given the differences in NK cell responses that have been shown to exist between C57BL/10 and SJL strains of mice, we had hypothesized that the degree of cytolysis by splenic NK cells would correlate with susceptibility (Paya et al., 1989). This was not the case as we found that CBA mice displayed more cytolytic reactivity than BALB/c mice. In contrast, SJL mice displayed almost no detectable NK activity.

Our findings support those of others who have shown a negative effect of chronic stress on the NK cell response in both mice (Sheridan et al., 1998; Hunzeker et al., 2004; Welsh et al., 2004; Tseng et al., 2005) and humans (Herbert and Cohen, 1993; Cohen et al., 2007). The mechanism by which RS reduces NK cell function has been partially explained in mice, and involves both the SNS and the HPA axis. For instance, during influenza infection, the actions of the SNS were shown to mediate the effects of RS on

cytotoxicity, whereas the HPA axis caused decreased NK cell trafficking (Tseng et al., 2005).

Despite these effects, we failed to find a significant correlation between decreased function in the spleen and increased CNS viral titers, which may suggest differences in NK numbers or responsiveness between the site of infection (CNS) and the periphery (spleen). Alternatively, this finding could implicate other innate immune functions in limiting replication of TMEV within the CNS during early infection that are suppressed by RS.

One possible mechanism by which viral replication could be limited at 24 hours post infection is through the production of the type 1 interferons, IFN-α and IFN-β. The type 1 interferons are rapidly induced following infection, and almost all cells are capable of their production, including cells within the CNS. For instance, in TMEV as well as Le Crosse virus infection, it has been shown that both IFN-β and some forms of IFN-α (particularly the α5 and α2 isoforms) can be secreted by neurons (Delhaye et al., 2006). Additionally, type 1 interferon mRNA expression has been shown to be up-regulated following infection of microglia with TMEV *in vitro* (Olson et al., 2001). Importantly, these proteins increase expression of MHC class I in the CNS of TMEV-infected mice as early as 6 hours post infection, which may facilitate clearance of virus by $CD8^+$ T-cell mediated processes that occur at later time points (Njenga et al., 1997). Finally, the importance of IFN-α/β production in protection from TMEV as well as other encephalogenic viral infections, has been demonstrated in IFN-$\alpha/\beta^{-/-}$ mice (van den Broek et al., 1995).

It is conceivable that the inhibition of such an early response within the CNS by stress could account for increased viral titers demonstrated at this time point. Contrary to this hypothesis, RS was not found to decrease either mRNA expression of IFN-α or IFN-β at day 1 p.i., but was shown to augment type 1 interferon mRNA expression of both the lung during at day 3, 5 and 7 p.i. with influenza virus infection (Hunzeker et al., 2004). Similarly, Mi et al., (2006) found that while neither stress nor infection significantly altered mRNA expression of IFN-β in the CNS at day 2 p.i., within infected mice, RS resulted in a significant increase in this interferon by day 7 p.i. Thus, RS may lead to increases in type 1 interferon production as a compensatory mechanism for protection against viral infection (Hunzeker et al., 2004). Alternatively, the RS induced increase in type 1 interferons could mirror increased viral titers by stress demonstrated at these times (Mi et al., 2006a). Taken together, these studies suggest a requirement for type 1 interferon production in limiting TMEV infection, but they strongly indicate that the mechanism by which RS causes increased viral titers in our experiment was not due to a decrease in this innate response because the association with an NK cell-mediated effect on viral clearance was best observed in susceptible SJL mice which have a defective development of NK cells (Fig. 2-3A; Kaminsky et al., 1987). Important to this argument is the fact that viral titers were not influenced by RS in this mouse strain (Fig. 2-3C).

Given the protective effects of IFN-γ in TMEV clearance from neurons within the CNS (Rodriguez et al., 2003), and our inability to detect IFN-γ in the serum of mice at day 1 p.i., it becomes important to investigate further the effects of RS on the role of

NK-cell mediated IFN-γ secretion and number of IFN-γ producing NK-cells within the CNS, and its relation to TMEV viral titers. To our knowledge the effects of RS on this important aspect of NK-cell effector function has yet to be investigated in a viral model of disease. However, Mi et al., (2006) have produced some indirect evidence that suggest a stress-induced decrease in this response. Specifically, they were able to demonstrate that at day 2 p.i. with TMEV, mRNA expression of IFN-γ was increased in the CNS of infected animals, and that this appeared to be slightly decreased in stressed mice (Mi et al., 2006b). In addition, analysis of mRNA expression of IFN-γ from spleen cells taken at day 2 p.i., was found to be decreased by RS (Mi et al., 2006a). Because NK cells, $CD4^+$, and $CD8^+$ T-cells are the major producers of IFN-γ, the time-frame of this effect is suggestive of a RS-mediated decrease in NK cell production of IFN-γ. However, in these studies it is impossible to rule out DC, or NKT cells as the effector cell(s).

3. Effects of RS on T-cell mediated immunity toward TMEV

Although innate immunity contributes to viral limitation during early disease, it is the abrupt generation of a robust virus-specific adaptive immune response that impedes TMEV persistence and subsequent demyelination. Thus, functional $CD4^+$ T-cell (Welsh et al., 1987; Lin et al., 2004; Karls et al., 2002), $CD8^+$ T-cell (Mendez-Fernandez et al., 2003; Begolka et al., 2001; Delthefs et al., 1997), and antibody responses (Kang et al., 2005) are an absolute necessity for TMEV clearance. Indeed, Mohindru et al., (2006) have recently demonstrated that prior immunization of

susceptible SJL mice with the $CD4^+$ T-cell immunodominant peptides ($VP1_{233-250}$, $VP2_{74-86}$, and $VP3_{24-37}$) results in delayed disease onset. Alternatively, susceptible CBA mice depleted of $CD4^+$ T-cells by thymectomy and antibody die shortly after infection (Welsh et al., 1987).

The effects of $CD8^+$ T-cells on TMEV clearance are best illustrated by the results of Delethfs, Mendez-Fernandez, and Lyman wherein they show that an early (Delethfs et al., 1997), but robust (Lyman et al., 2004) $CD8^+$ CTL response to a single immunodominant epitope is required (Mendez-Fernandez et al., 2003). Interestingly, it has also been hypothesized that the $CD8^+$ T-cell response can account for the differences in the severity of DA versus BeAn strains of TMEV infection in SJL mice, as DA, produces a more severe form of early disease and contains one $CD8^+$ T-cell dominant epitope, whereas the BeAn strain contains three $CD8^+$ T-cell immunodominant epitopes and produces a less severe form of acute disease (Kang et al., 2002b).

The above studies show that in SJL mice, the virus-specific cellular response is compromised by stress. Specifically, we demonstrated that the number of IFN-γ producing cells in the CNS of I/R mice was reduced by nearly 60% when compared to I/NR mice. These cells were determined to be almost exclusively $CD8^+$ T-cells as indicated by their ability to recognize the MHC class I restricted peptide $VP3_{155-166}$, and their inability to recognize the MHC class II restricted immunodominant peptide $VP2_{74-86}$, a finding that confirms those of Mohindru et al., (2006).

Moreover, we also found that the amount of IFN-γ secreted in response to stimulation with whole UV-irradiated virus, was severely diminished in stressed mice.

This finding was observed in the spleen as well as the CNS. In addition, cytokine measurements from CNS-IL isolated at day 8 p.i., revealed increases in virus-specific IL-17A. This finding is important as it may indicate that the majority of $CD4^+$ T-cells present in the CNS during early infection with TMEV are virus-specific IL-17A producing cells. These findings are also the first to suggest that restraint stress can decrease IL-17A responses within the CNS.

The observed immunosuppressive effects of stress, particularly IFN-γ responses within the CNS, are likely the cause of increased viral titers observed during this time-frame (Mi et al., 2006b). The mechanism by which stress ameliorated TMEV-specific T-cell responses was partially attributable to the actions of glucocorticoids on virus responsive $CD8^+$ T-cell numbers. Indeed IFN-γ producing $CD8^+$ T-cell numbers were significantly restored in the spleen and showed a strong trend toward restoration within the CNS following concurrent RU486 treatment. This finding differed from studies investigating the effects of restraint stress on influenza pathogenesis that demonstrated complete restoration of IFN-γ production after stimulation with whole influenza virus (Dobbs et al., 1996).

These discrepancies may be explained by the fact that the above study utilized viable virus as a stimulus over the course of 72 hours which is likely to have resulted in IFN-γ production from NK cells present in their cultures (Dobbs et al., 1996). In contrast, we used viral peptides designed to specifically target either $CD4^+$ or $CD8^+$ T-cells. Furthermore, SJL mice lack functional NK cells (Fig. 2-3A), thus ruling out the possibility that NK cells would be the source of IFN-γ. In support of this hypothesis,

levels of IL-2, a cytokine not produced by NK cells, was not completely restored in mice that received restraint stress and concurrent administration of RU486 (Dobbs et al., 1996). Nevertheless, these studies together indicate a role for glucocorticoids in mediating suppression of type 1 immune responses during viral infection. However, due to the partial restoration of cytokine production seen in our studies, the role that the SNS plays in cytokine production in stressed mice should also be considered.

In general, stress-induced increases in glucocorticoids may have caused decreased immunity behind the BBB in several ways. For instance, stress may have caused T-cell induced apoptosis, possibly resulting in substantially decreased T-cell numbers. Alternatively, stress-induced glucocorticoids may have caused decreased trafficking of T-cells to the CNS, or stress may have had a direct effect on effector function at the T-cell level. Finally, stress may have affected the generation of T-cell responses through the actions of glucocorticoids on antigen presenting cells.

3.1 Suppression through lymphopenia

The hypothesis that stress caused T-cell induced apoptosis is supported by the reduction of both thymus and spleen mass following restraint stress (Campbell et al., 2001). This finding was replicated in our studies by the observed reductions in the total number of cells isolated from the spleens restrained mice (Fig. 3-11B). While this is a legitimate hypothesis, it does not explain the reductions in effector function that we observed when we normalized for cell number in our cultures. A reduction in T-cells would have supported the hypothesis that many of our effects were attributable to

differences in cell population. Flow cytometric analysis of T-cell percentages, demonstrated only small effects of stress on $CD4^+$ and $CD8^+$ T-cell populations, and while there was a significant decrease in $CD4^+$ T-cell percentage, stress the $CD8^+$ T-cell percentages actually increased. As such, these small alterations are not likely account for our observed effects.

3.2 Suppression through altered trafficking

Suppression of virus-specific immune responses within the CNS of stressed mice could have been brought about by decreases in local proliferation, increased efflux from the CNS, increased cell death within the CNS or decreased trafficking to the CNS. The suppression of IL-2 production by virus-stimulated CNS-ILs taken from infected stressed mice may suggest decreases in local proliferative responses. While stress may have caused increased T-cell efflux or cell death, the evidence that stress could have caused decreases in T-cell trafficking to the CNS is noteworthy for several reasons: first and foremost, we observed decreased numbers of virus-specific cells within the CNS of stressed mice. Secondly, stimulation of CNS-ILs using α-CD3/CD28 antibodies demonstrated suppression in nearly all cytokines within the CNS, possibly reflecting fewer T-cells numbers. Thirdly, these findings are supported by reductions in lymphocytes in the blood of stressed mice (Campbell et al., 2001; Zhang et al., 1998; Ottaway and Husband, 1994; Mizobe et al., 1997). Finally, the influence of restraint stress on lymphocyte trafficking is suggested by studies investigating the effects of stress on chemokines (Mi et al., 2004).

The chemokines RANTES and MCP-1 are important in the chemoattraction of both memory T-cells and monocytes, respectively to the site of infection. In TMEV infection, these chemokines have been proposed to aid in early viral clearance from the CNS, but are also thought to contribute to the pathogenesis of demyelination in the chronic stages of infection by sequestering autoreactive lymphocytes behind the blood-brain barrier (Murray et al., 2000; Ransohoff et al., 2002; Kim et al., 2005). Our results agree with those of Mi et al., (2004) which indicate that restraint stress decreases circulating levels of these chemokines at a time when cellular trafficking into the CNS is critical for viral clearance. Mechanistically, these effects could be, in part, due to decreases in IFN-γ, which has been shown to stimulate the production of RANTES and MCP-1 (Valente et al., 1998). To this end, it is important to mention that in our study, serum concentrations of IFN-γ were positively correlated with those of RANTES and MCP-1 (not shown).

While RS decreased the serum concentrations of most cytokines and chemokines, this was not the case for the chemokine KC (CXCL1) and growth factor G-CSF. Both KC and MIP-2 (CXCL2) are thought to function as mouse homologs of human IL-8. As such, these CXC chemokines play a major role in the trafficking of neutrophils. The hematopoetic factor G-CSF is, in part, responsible for the maturation of neutrophils from the bone marrow, and indeed is used clinically to bolster circulating neutrophils in patients undergoing chemotherapy. RS dramatically increased serum concentrations of these proteins (Table 3-3). These results build on our previous findings (Campbell et al.,

2001; Welsh et al., 2004) by suggesting that it is the modulation of KC and G-CSF by RS that contribute to RS-induced neutrophilia.

Unlike RANTES and MCP-1, KC gene transcription is down regulated by IFN-γ (Ellis and Beaman, 2004). While IFN-γ concentrations did not significantly correlate with either KC or G-CSF concentrations in this study, we showed that TMEV infection decreased serum levels of KC, but not G-CSF. These results, obtained from the periphery, are in contrast to what is observed in the CNS of susceptible mice as it has been shown that TMEV infection can increase both KC and MIP-2 expression in primary astrocytes from susceptible mice and humans (Rubio et al., 2006). A study by Rubio et al. (2006) suggests that MIP-2 may contribute to the pathogenesis of TMEV-induced demyelination, as astrocytes from susceptible (SJL) but not resistant mice (BALB/c) secrete MIP-2. Interestingly, roles for KC and G-CSF in the pathogenesis of demyelinating disease have also been illustrated in the experimental allergic encephalomyelitis (EAE) model of human multiple sclerosis (Tran et al., 2000; Verda et al., 2006). Specifically, Tran et al. (2000) demonstrated that IFN-$\gamma^{-/-}$ or IFNγR$^{-/-}$ BALB/c mice are rendered susceptible to MBP-induced EAE and produce high levels of KC. Pathologically, the inflammatory infiltrate in the CNS was comprised of mostly neutrophils and was localized to areas of demyelination (Tran et al., 2000). While these results suggest that neutrophils are actively involved in the demyelination process of both animal models of MS, a subsequent study using the EAE model suggested that infiltrating neutrophils serve a regulatory role through the secretion of nitric oxide, and that this regulatory function is in part dependent on IFN-γ (Zehntner et al., 2005).

Additionally, Verda et al. (2006) have recently demonstrated that G-CSF administration causes exacerbation of EAE (Verda et al., 2006). Alternatively, the increases in serum KC and G-CSF could be in part due to IL-17 production from T-cells (Witowski et al., 2004). In regards to the latter, it is interesting that while stress reduced the amount of IL-17A production within the CNS, antibody stimulation of T-cells in the spleen revealed NI/R mice produced more IL-17 than other groups, a finding that needs to be substantiated.

3.3 Suppression by direct action on T-cells

In general, cellular immune responses (Th1) are characterized by the secretion of IFN-γ and IL-2 from $CD4^+$ T-cells (Abbas et al., 1996). The generation of Th1 responses occurs through the presentation of antigen, costimulation, and secondary stimulation of naive T-cells with proinflammatory cytokines, particularly IL-12. Binding of active IL-12(p70), composed of the p35 and p40 subunits, to its receptor, stimulates activation of the Janus family of tyrosine kinase2 (JAK2) and TYK2 (Agnello et al., 2003; Bacon et al., 1995) which directly activates the signal transducer and activator of transcription (STAT)4 to translocate to the nucleus and induce the transcription of IFN-γ (Usui et al., 2006; Wurster et al., 2000). Upon secretion, IFN-γ can act in an autocrine or paracrine fashion. Binding of IFN-γ to its receptor causes phosphorylation and subsequent activation of STAT1, which results in the transcription of the T-box expressed in T cells (T-bet) transcription factor, the master driver of Th1 polarization (Afkarian et al., 2002; Szabo et al., 2000).

T-bet, a member of the family of T-box transcription factors, is expressed in $CD4^+$ and $CD8^+$ T-cells, NK cells, B-cells and DCs. The actions of T-bet have been discussed (Section III), but it should be reiterated that this transcription factor plays a critical role in the development of the Th1 response (Szabo et al., 2000). While ectopic expression of T-bet has been demonstrated to drive Th1 expression in cells that had been biased toward Th2 (Szabo et al., 2000), the effects of T-bet on the generation of a type 1 immune response is best illustrated by the phenotype of T-$bet^{-/-}$ mice. T-$bet^{-/-}$ mice produced sub-optimal amounts of IFN-γ (Lugo-Villarino et al., 2003; Szabo et al., 2002), have reduced NK cell cytotoxicity (Szabo et al., 2002), diminished T-cell activation by dendritic cells (Lugo-Villarino et al., 2003) decreased IL-12R expression (Afkarian et al., 2002), diminished antigen-specific $CD8^+$ T-cell-mediated CTL response (Sullivan et al., 2003), and reduced proinflammatory $CD4^+$ T-cell trafficking (Lord et al., 2005). The actions of T-bet on Th1 polarization, and IFN-γ gene transcription have recently been attributed to its direct binding (Hwang et al., 2005) and inhibition of the transcription factor GATA-3 (Usui et al., 2006).

Unlike Th1 immunity, Th2 immunity is characterized by the secretion of cytokines IL-4, IL-5, IL-10 and IL-13 from $CD4^+$ T-cells and by promoting the generation of antibody responses that are effective at reducing helminthic infections (Agnello et al., 2003). The generation of a Th2 response is dependent on IL-4 binding its receptor and the subsequent phosphorylation of STAT6 (Hou et al., 1994; Agnello et al., 2003). Active STAT6 can then translocate to the nucleus and induce the expression of GATA-3. Importantly, the transcription of the *Gata3* gene has recently been shown

to require both Notch 1 and 2 signaling and subsequent activation of RBP-J on the *Gata3* promotor leading to Th2 polarization by processes independent of IL-4 and STAT6 (Amsen et al., 2007). The actions of GATA-3 are opposite of T-bet, as it is necessary and sufficient for Th2 polarization (Zheng and Flavell, 1997). Once activated, GATA-3 can increase the gene transcription of Th2 cytokines by acetylating histones within their promotor regions (Avni et al., 2002). In addition, Usui et al., (2006) have recently demonstrated that GATA-3 activation inhibits STAT4 induced transcription of the *IFNG* gene. Therefore, the functions of the transcription factors T-bet and GATA-3 provide a molecular framework for understanding the Th1/Th2 paradigm as well as the generation of an effective immune response.

Recently, there has been described a new pro-inflammatory lineage of T helper cells, designated Th17, that plays an important role in the induction of EAE (Harrington et al., 2005; Park et al., 2005; Komiyama et al., 2006). The polarization of these cells is dependent on the synergistic actions of IL-6 and TGF-β on naïve T-cells, the phosphorylation of STAT3, (Chen et al., 2006; Yang et al., 2007) and the subsequent activation of the transcription factors RORγt and RORα (Ivanov et al., 2006; Yang et al., 2008). However, their propagation is dependent on IL-23 stimulation (Bettelli et al., 2006; Veldhoen et al., 2006; Mangan et al., 2006). In mice, IFN-γ acts to suppress both the induction and actions of these cells after mycobacterial infection indicating a regulatory role for IFN-γ on the effector function of these cells (Cruz et al., 2006). The knowledge of these molecular events is imperative for understanding how stress can effect the generation of Th1, Th2 and Th17 immunity to TMEV.

The ability of stress to inhibit effector function is supported by the fact that glucocorticoids have been shown to inhibit IFN-γ production of $CD8^+$ T-cells in HSV-1 infection, without influencing the number of $CD8^+$ cells within the CNS (Freedman et al., 2007). Similarily, we showed that glucocorticoid treatment inhibited both $CD4^+$ and $CD8^+$ specific IFN-γ secretion in splenocytes taken from I/NR mice. It is possible that these effects are due to the inhibitory actions of glucocorticoids on T-bet which has been shown to occur by direct binding of the activated glucocorticoid receptor to T-bet, resulting in decreased binding of this transcription factor to the *Ifng* promoter (Liberman et al., 2007). Glucocorticoids have also been demonstrated to inhibit the actions of STAT1 (Hu et al., 2003), STAT4 (Franchimont et al., 2000), GATA-3 (Jee et al., 2005), NFkB and AP-1 (Webster et al., 2002), strongly indicating a suppressive role at the T-cell level.

3.4 Suppression through decreased generation of T-cell responses

Our findings can also be explained by the failure to generate an immune response to TMEV, which may reflect a stress-induced impairment of antigen presenting cell function. Supporting this hypothesis are data that clearly indicate suppressive effects of glucocorticoids on dendritic cells (DCs). In these studies, it was found that DC treated with glucocorticoids had altered processing and presentation of MHC class I and class II molecules as well as decreased costimulatory molecule, particularily CD80/CD86, expression on their cell surface, as well as decreased IL-6, IL-12 and TNF-α production after activation with LPS (Truckenmiller et al., 2005; Elftman et al., 2007). This

mechanism for immunosuppression is supported by the decreased circulating serum levels of IL-12 (p40) and IL-12 (p70) in stressed mice. Additionally, the decreased responses to the T-cell stimulatory antibodies could also be perceived as supportive of this concept as they may indicate fewer T-cell clones rather than total number of cells. In this regard, the reductions in T-cell responses were also observed in the spleens of stressed mice.

3.5 Failure of stress to effect C57BL/6 immunity

Taken together, we demonstrate that in SJL mice, restraint stress greatly decreases both $CD4^+$ and $CD8^+$ T-cell responses within the CNS regardless of the mechanism. However, it is worth mentioning that in C57BL/6 mice this was not found to be the case. In fact, there were no readily observed effects of RS on the CTL responses to TMEV infected CVE cells. The inability for stress to alter CD8 responses to TMEV was reflected in both the viral titers and the unaltered susceptibility to the late demyelinating disease, as indicated by histological analysis (Fig. 4-7B).

The reason for the discrepancies in the ability of restraint stress to alter immune responses to TMEV between SJL and C57BL/6 mice remains unknown, however several possibilities exist, most of which have received attention (Section IV). One previously unmentioned, but particularly interesting, possibility is the fact that different responses to chronic stress have been shown to exist between individual mice within the C57BL/6 strain (Krishnan et al., 2007). Using social defeat stress as a model, these authors were able to show that increased brain-derived neurotropic factor (BDNF) resulting from

hyperactive ventral tegmental area (VTA) neurons resulted in susceptibility to social defeat. Susceptibility to defeat was indicated by decreased sucrose preference, hypothermia, body mass, and circadian amplitude (among others) compared to controls, but was not reflected by altered serum glucocorticoids levels (Krishnan et al., 2007). Given the relatively small number of mice used in our study, the above study raises the possibility, although unlikely, that all of the mice in the stressed group were non-suseptible to stress. Noteworthy, is our observed reduction in body mass within our stressed mice, which in the above study was an indicator of susceptibility (Krishnan et al., 2007). A more probable explanation is that the immune response toward TMEV requires longer lasting restraint sessions to achieve immunosuppression (Sheridan et al., 1998), a procedure that, due to age restrictions of TMEV infection, resulted in high incidence of morbidity and mortality in preliminary studies making it unfeasible.

4. RS differentially affects humoral immune responses to early TMEV infection

The observation that stress raised virus-specific serum antibody levels in SJL mice was unexpected given the degree of virus -specific $CD4^+$ T-cell immunosuppression that was demonstrated in the spleens of infected mice (Fig 3-5). Importantly, this infliction by stress was not attributed to a shift towards Th2 immune responses as suggested by many (for review see Webster et al., 2002; Elenkov, 2004; Calcagni and Elenkov, 2006). Indeed, both type 1 and type 2 responses were suppressed, but not all cytokines were suppressed. In fact, despite decreases in serum levels of IL-4, and IL-5 by restraint stress, IL-6 levels, which have potent B-cell

stimulatory properties, were increased in the serum by stress (Tables 3-1 and 3-2). Given the overall immunosuppressive action of restraint stress on stimulated T-cells, it is likely that the increase in serum IL-6 was derived from an alternate source. The demonstration of increased serum levels may implicate hepatocytes as the culprit, but none of the other many cellular sources of IL-6 should be ruled out. While the mechanism for stress- induced antibody production is not yet clear, it is very likely to be under genetic control, as C57BL/6 mice that underwent similar restraint stress sessions displayed slightly reduced, but non-significant decreases in virus specific antibody levels (Fig. 4-3). Interestingly, a recent study by Hou et al., (2007), has indicated that DCs from SJL mice produce much higher levels of IL-6 then DCs from C57BL/6 mice when infected with TMEV. This finding however, cannot be applied directly to the current studies, as we demonstrated a strong trend for infection to reduce the augmented effects of stress on IL-6 levels, which indicate a suppressive role for infection. To this end, the differential effects of stress on IL-6 production in SJL and C57BL/6 strains have not yet been compared. However it is interesting that the administration of recombinant IL-6 to TMEV-infected SJL mice has been shown to result in increases in TMEV-specific anitbody production, which is protective upon transfer to infected mice (Rodriguez et al., 1994), and suggests that IL-6 is capable of playing a mechanistic role in the upregulation of TMEV-specific antibody responses that are protective against early encephalitis.

Interestingly, stress-induced increases in TMEV-specific antibodies did not appear to be attributable to the actions of glucocorticoids, as administration of dexamethasone was found to decrease antibody levels while treatment with RU486 did

not reverse this effect. These results contrast with those of early *in vitro* studies that indicate a necessity and sufficiency of the suppressive actions of glucocorticoid on production of antibody to both T-cell independent (P-PAA) and T-cell dependent (SRBC) antigens (Emilie et al., 1984). This strongly implies that other mechanisms, such as those mediated by the SNS, are involved. With respect to this hypothesis, it is interesting that B-cells, DCs and Th1 cells, but not Th2 cells, have been demonstrated to posses β2-adrenergic receptors (ARs), indicating that both epinephrine and norepinepherine can directly influence these cell types (Elenkov et al., 1996; Johnson et al., 2005; Kavelaars et al., 1997; Mohamed-Ali et al., 2001).

The studies herein are not the first to report altered T-cell responses coupled to unaltered virus-specific serum antibody levels (Sheridan et al., 1991). Moreover, the increases in antibody levels in the serum are not necessarily indicative of increased ability to clear viral infections (neutralizing). For instance, caretakers of dementia patients have been shown to have increased circulating levels of anti-HSV-1 antibodies, when compared to non-caretakers, but at the same time did not reflect an increase in neutralizing titers (Glaser and Kiecolt-Glaser, 1997). Furthermore, even if the increased TMEV-specific antibody levels in SJL mice were neutralizing, their circulation in the serum is not beneficial to limiting viral spread behind the blood-brain barrier between neurons. In this regard, it is important to reiterate that we could not detect any virus-specific-antibody-secreting cells within the CNS at either days 8 or 16 p.i. In contrast, we observed copious numbers of virus-specific antibody-secreting cells within the CNS of infected mice during late infection, but relatively few in the spleens of these mice (Fig

5-1). The latter findings are supported by recent studies which more thoroughly characterize the production of intrathecal antibody (Pachner et al., 2007a; 2007b). Nevertheless, if these antibodies are found to be neutralizing, a means of entry into the CNS must be considered as circulating antibody are unable to cross the BBB.

Entry of circulating anti-viral antibodies into the parenchyma of the CNS could be facilitated in several ways: First, viral infection of either cerebrovascular endothelial cells or astrocytes, the cells which comprise the BBB, would be expected to compromise the integrity of the BBB allowing leakage of antibody. Secondly, stress may actually aid in the diffusion of antibody from the circulation into the CNS by opening the BBB. In support of this mechanism, acute restraint stress in rats has been shown to open the BBB, a process that was dependent on activation of mast cells and subsequent degranulation and histamine release in response to exposure of CRH (Esposito et al., 2001; Esposito et al., 2002). While the effects of chronic restraint stress on this occurrence has not been thoroughly investigated, it has been shown repeatedly to increase serum corticosterone (Sieve et al., 2004; 2006; Fig. 4-5C) , a down stream product of HPA axis activation, and preliminary studies from our laboratory suggests that the BBB is, in fact, compromised following chronic restraint stress. These possibilities should be the subject of future investigation.

The increase in virus-specific antibody levels by stress in SJL mice may also play a role in the pathogenesis of demyelination, a possibility that should not be readily dismissed. For instance, as occurs in MS, TMEV infection has recently been shown to

result in intrathecal antibody production (Pachner et al., 2007a) that begins around the time of demyelination, and is in fact correlated with disability and viral titers (Pachner et al., 2007b). The percentage of antibody-secreting cells specific for TMEV in the CNS during late disease is suspected to be around 54% compared to 8% in the spleen (Pachner et al., 2007b and Fig 5-1). Even though T-cells obviously play a role in clearance of TMEV from the CNS, they are also facilitators of demyelination in late disease (Lipton, and Dal Conto, 1976; Welsh et al., 1987; Rodriguez et al., 1986). This T-cell mediated destruction of myelin in the late disease, may be enhanced by humoral responses behind the BBB. One mechanism by which this could occur is through complement-mediated lysis of oligodendrocytes. Indeed, while the CNS is considered immunoprivileged, it possesses all of the components of the complement cascade. Alternatively, autoantibody binding to myelin could opsonize oligodendrocytes allowing microglia or infiltrating monocytes to destroy the tissue by phagocytosis. Finally, antibody could cause direct lysis of cells by antibody-induced cellular cytotoxicity (Antel and Bar-Or, 2006).

The fact that only 54% of antibody producing cells during chronic disease are TMEV specific, indicates that roughly 40-50% recognize alternate antigens. Importantly, several studies have demonstrated the presence of anti-myelin antibodies in the serum of TMEV-infected mice during late disease (Rauch et al., 1987; Sieve et al., 2004; Sieve et al., 2006). Of particular interest is the development of antibodies directed against the protein myelin oligodendrocyte glycoprotein (MOG), a small tansmembrane glycoprotein that makes up approximately 0.02-0.05% of total myelin. Despite its low

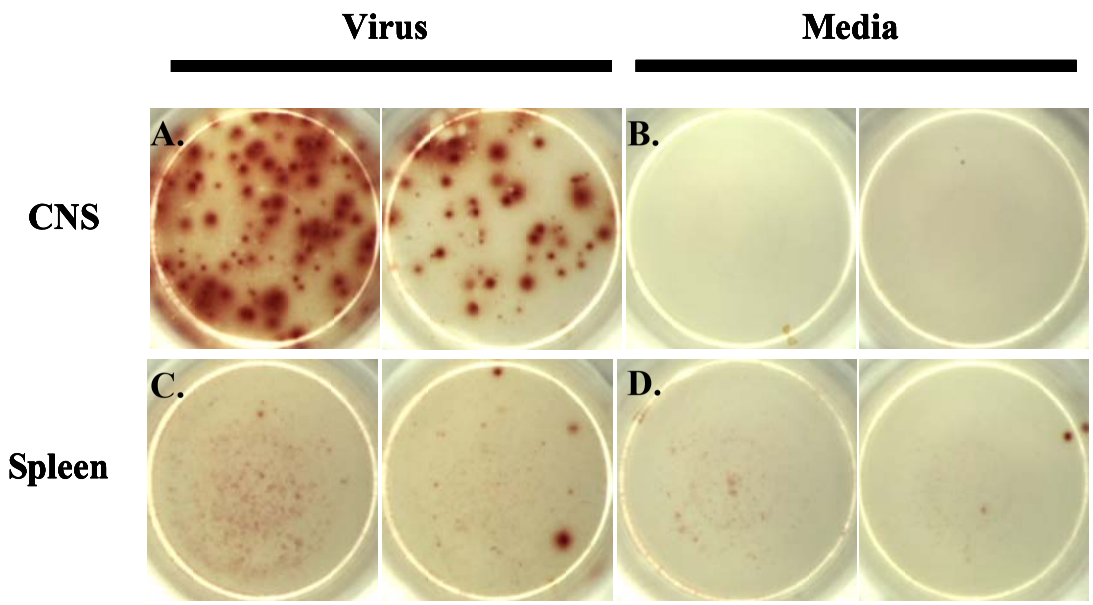


Figure 5-1. Occurrence of virus-specific antibody producing cells in the CNS and spleen during late disease. SJL mice (n = 2) were infected with BeAn 8386. At day 74 p.i. CNS-IL (1.0×10^5; top row) or spleen cells (1.0×10^6; bottom row) were plated in wells previously coated with purified virus (left) or media (right) for 24 h at 37° C and 5.0% CO_2. The plate was incubated with biotinylated anti-mouse IgG + IgM for 2 h at room temp., followed by avidin-HRP for 30 min. Spots were developed with AEC. (A). Virus-specific antibody producing cells from CNS. (B) Respective media controls for samples in A. (C). Virus-specific antibody producing cells from spleens. (D). Respective media controls for samples in C. Results are derived from same mice. Representative singlet samples from each mouse are shown.

content in myelin, MOG is a relevant target for antibody binding and subsequently the subjection of much study in human MS (Reindl et al., 2006). This is because MOG contains an Ig domain that is completely exposed to the extracellular environment. In fact, MOG peptides have been shown to be encephalogenic, causing EAE in rats, mice, rhesus monkeys, and marmosets (Iglesia et al., 2001). Moreover, EAE induction in both rats and marmosets requires the presence of specific B-cells, strongly indicating a role for antimyelin responses in the development of EAE (Iglesia et al., 2001). Importantly, the presence of circulating anti-MOG (MOG_{35-55}) antibodies during the chronic phase of TMEV infection of SJL mice has also been demonstrated possibly indicating a parallel mechanism of tissue destruction between animal models and human disease (Sieve et al., 2004). Our results, in SJL mice, present the possibility that stress, either by increasing the overall humoral response, or by allowing entry of autoreactive antibody into the CNS by disruption of the BBB could modulate the demyelination process.

5. Conclusion

Pathologically, the hallmark of MS is demyelination, which occurs in the white matter of the CNS, usually in a perivascular fashion around post-capillary venules and veins and is thought to result from immune-mediated destruction of myelin. The demyelinated 'plaque' displays heterogeneity, and has been sub-typed to fit one of four categories based on the pattern of demyelination (Lucchinetti et al., 2000). All four types of plaques (I-IV) contain T-cells and macrophages. However, Lucchinetti et al., (1999;2000) describe the type I lesion as being macrophage associated as it resembles

the models of demyelination resulting from TNF-α and reactive oxygen species; the type II lesion is also macrophage associated but additionally contains deposition of immunoglobulin and activated complement; the type III lesion resembles demyelination mediated by apoptosis and is reminiscent of demyelination that occurs following some viral infections of oligodendrocytes; the type IV lesion represented non-apoptosis associated primary oligodendropathy (Lucchinetti et al., 1999; 2000). This heterogeneity along with additional studies conducted in both MS patients as well as in animal models of MS, particularly experimental autoimmune encephalomyelitis (EAE), suggests that T-cells are responsible for the pathogenesis of MS (Chitnis, 2007).

Historically, MS has been considered the prototypical Th1-mediated autoimmune disease which is mediated by trafficking of autoreactive cells from the periphery into the CNS (Sospedra and Martin, 2005). Reasons to suspect autoimmune $CD4^+$ T-cells in the pathogenesis of MS include, but are not limited to: the presence of $CD4^+$ T-cells in all four types of lesions (Lucchinetti et al., 1999; 2000); the increased prevalence of active MBP specific T-cells in the periphery and cerebrospinal fluid of MS patients vs. controls (Zhang et al., 1994); the exacerbation of disease in patients following treatment with altered peptide ligand mimicking MBP (Bielekova et al., 2000) the unfavorable response following treatment of patients with IFN-γ (Panitch et al., 1987); the robust genetic association with the HLA-DR locus (Hafler et al., 2007); the occurance of polymorphisms in genes involved in immunoregulation of T-cells in MS patients (Chitnis, 2007); marked decrease in disease activity following treatment with a humanized monoclonal targeting $\alpha_4\beta_1$integrin (natalizumab; Miller et al., 2003); the

spontaneous development of EAE in transgenic mice on *Rag2*$^{-/-}$ made to express both the human HLA-DR*1501 as well as TCR isolated from T-cell clone of an MS patient (Madsen et al., 1999); and the ability of myelin specific CD4^{+} T-cells to induce EAE following adoptive transfer into naive mice. However, Th1 cells may not be as exclusively involved in MS pathogenesis as once thought since Th17 cells have recently been implicated. In light of this evidence, it is still imperative to consider other mechanisms of demyelination as anti-CD4 therapy in MS patients was demonstrated to effectively delete CD4^{+} T-cells, but was not able to effectively alter disease course (von Oosten et al., 1997).

Other immune cells are also thought to play a role in the pathogenesis of MS, although the mechanisms have not been as intensively researched. Supporting evidence for a CD8^{+} T-cell involvement includes, but is not limited to: the fact that CD8 T-cells consistently outnumber CD4^{+} T-cells within the lesion by at least 2:1; CD8 memory T-cells have been shown to be clonally expanded in the CNS of MS patients (Bebbe et al., 2000); class I MHC can be expressed on electrically silent neurons or functionally impaired neurons (Neumann et al., 1995); the transfer of myelin specific CD8^{+} T-cells into naive mice can induce EAE (Huseby et al., 2001); and there is an association between HLA-A0201 and MS (Fogdell-Hahn et al., 2000). However, the involvement of HLA-A0201 in the pathogenesis of MS is somewhat controversial as both protective and detrimental effects have been described (Sospedra and Martin, 2005; Fogdell-Hahn et al., 2000). Additionally, B-cells are thought to play a role in the pathogenesis of MS. The importance of B-cell involvement in MS is illustrated by the deposition of antibody

and complement in the type II lesion described above; the prevalence of oligoclonal bands in the CSF of most MS patients indicating intrathecal antibody production and clonal expansion; the suspected correlation between circulating anti-MOG and anti-MBP Ig at onset with disease exacerbation as determined by MRI (Berger et al., 2003). Also, treatment with monoclonal antibodies (rituximab) directed against the B-cell marker CD20 provides some promising therapeutic results in the ability to MS patients (Muraro and Bielekova, 2007).

Innate immune responses, other those associated with macrophages and dendritic cells, are also thought to influence the pathogenesis of MS. For instance, the activation and degranulation of mast cells, which have been identified within the MS plaque, could result in opening the BBB; the abundant production of IFN-γ by NKT cells in MS patients could influence macrophage and microglia activation as well as MHC class I expression on glial cells; γδ T-cells are present in chronic lesions of MS patients, and have demonstrated the capacity to lyse oligodendrocytes in a perforin-dependent fashion (reviewed in Sospedra and Martin, 2005). In terms of the latter, it is interesting that one study indicated the capacity of γδ T-cells to act as professional antigen presenting cells (Brandes et al., 2005). Therefore, upon activation these cells may transverse the BBB and act as APCs within the CNS parachyma. On the other hand NK cells, especially those displaying the CD56 bright or CD95+ phenotype, appear to be regulatory and are thought to confer protection against the pro-inflammatory anti-myelin T-cell responses occurring in MS (Bielekova et al., 2006; Takahashi et al., 2004).

If stress is immunosuppressive then it should protect against autoimmunity. For instance, in EAE, the prototypical autoimmune disease, chronic stress is invariably beneficial, as illustrated by its delay of onset (Levine et al., 1962; Correa et al., 1998; Griffin et al., 1993). In fact, in some models of relapsing-remitting EAE, it has been suggested that the stress of paralysis on the animal is partially responsible for remissions (Levine and Saltznan, 1987).

Translationally, the effects of stress on human multiple sclerosis should also be beneficial. To this end, glucocorticoids are substantially beneficial when administered to patients during relapse, and speed recovery (Frohman et al., 2006). However, Li et al. (2004), convincingly demonstrate that chronic stress significantly increases the risk for acquiring MS. This conundrum may be explained by separating the events that occur during onset of MS and those that are occurring during relapse. Even though MS is idiopathic, much evidence points to an infectious agent as an initiating event. If the disease process in MS is caused and perhaps perpetuated by a viral agent, then chronic stress, through immunosuppression, might modulate the susceptibility to the disease.

In humans, the prototypical example of immunosuppression leading to demyelination is the disease progressive multifocal leucoencephalopathy (PML) which is caused by the ubiquitous JC virus. While approximately 80% of the population is seropositive for JC virus, immunosuppression is thought to be a key prerequisite for the development of PML, allowing uncontrolled viral infection of oligodendrocytes, which results in rapidly progressing virus-mediated demyelination (Sabath and Major, 2002). Similar to JC virus, Theiler's virus is a widespread infectious agent in mice (Lipton et

al., 2001), but in nature, rarely results in demyelination (Sabin and Olitsky, 1938). Most importantly, demyelination in TMEV is immune-mediated, but completely dependent on the ability of the virus establish a persistent infection.

Our results on the effects of stress during early Theiler's virus infection begin to establish a conceptual framework for how chronic stress can influence the onset of MS. If the autoimmune nature of MS is initially brought on by an infectious trigger, the translation of our results suggests that stressful life events in genetically susceptible individuals may heighten the risk for developing MS. At the same time, our results indicate that in genetically resistant individuals, chronic stress may not alter the risk for developing the disease.

6. Future Studies

6.1 Determine the influence of stress on the ability of NK cells to modulate early immune responses to TMEV

6.1.1 Determine the role and mechanism of stress on NK cell cytokine secretion

The effects of both acute and chronic stress on NK cell cytotoxicity has been extensively investigated in both humans and mice. In mice, the suppression of cytotoxicity is mediated through the SNS. However, cytokine production, in particular the production of IFN-γ, from purified NK cells has not been investigated. Importantly, the induction of cytotoxcity and cytokine production in NK cells has been attributed to two separate signaling pathways (Vivier et al., 2004). As such, it is conceivable that while RS may act via the SNS to inhibit cytotoxicity, stress could inhibit cytokine

production (IFN-γ) by similar means, through the HPA axis, or not at all. Studies employing the use of HPA agonist (corticosterone or dexamethasone) and antagonist (RU486) as well as catecholamine agonist (epinephrine or norepinepherin) and antagonist (propranolol) as treatments to address IFN-γ secretion from purified NK cells isolated from stressed or non-stressed mice, should begin to address this issue. Follow-up *in vitro* experiments using infected, non-stressed mice should be conducted that are intended to assess the phosphorylation status of proteins such as ZAP70, SHP1/2, as well as transcription factors known to induce IFN-γ transcription, which should be determined by qRT-PCR over a time course. In these experiments it will be important to utilize neutralizing antibodies to IL-15, IL-18, and IL-12 as these have been shown to induce IFN-γ transcription in NK cells and may influence the interpretation of the data.

6.1.2 Determine the effects of stress on the trafficking of NK cells to the CNS during TMEV infection

The demonstration of NK cells within the CNS during TMEV infection has been implicated by one particular study investigating the effects of CTL responses on viral clearance, whereby the investigators were able to demonstrate that percent cell lysis using CNS-IL from day 7 p.i. was decreased when NK cells were previously depleted (Lindsley et al., 1991). However, the amount of IFN-γ production from these cells was not determined. As such, the degree by which NK cells influence the development of type 1 immune responses in the periphery, as well as IFN-γ-mediated viral clearance in the CNS during early infection, and the potential compromise of this function by stress-

induced inhibition of NK cell trafficking to the CNS, has yet to successfully be demonstrated. While FACS analysis utilizing intracellular cytokine staining techniques would specifically indicate the number of NK cells producing IFN- γ cells in the CNS, the numbers of mice required to obtain enough cells would present a problem, especially when considering the need to counter balance across groups during the experiment (N = 20 per day for at least 3 days per time point). The use of IFN-γ ELISPOT assays incorporating YAC-1 cells as stimulators may aid in the detection of small numbers of NK cells isolated from the CNS during early infection, and may provide a more practical approach, providing that preliminary studies would result in IFN-γ production that is NK cell specific. Additionally, restraint stress could cause decreases in IFN- γ production from NK cells in a variety of different fashions. For instance, RS has been shown to decrease mRNA levels of IL-15, and IL-12 (Hunzeker et al., 2004) potentially influencing both the survival and proliferation of NK cells as well as the transcription and secretion of IFN-γ. These effects should also be addressed.

6.2 Determine the immunological relevance of restraint stress induced increases in antibody titer and its influence on the pathogenesis of TMEV infection

6.2.1 Immunologic relevance of increased antibody in stressed and non-stressed mice

As mentioned, increases in virus-specific antibody levels are not always indications of increased immunity (Glaser and Kiecolt-Glaser, 1997). Therefore, the level of neutralizing titers of serum antibody should first be determined in order to speculate on the potential of antibody to limit viral spread within the CNS. Because

there is no guarantee that circulating antibody will reach the site of infection (CNS parenchyma) experiments should be conducted in I/NR, I/R, NI/NR, and NI/R mice to determine the degree of antibody allowed entry into the CNS of these mice, as stress may result in a compromised BBB. The amount of antibody reaching the CNS could be measured in several fashions. One potential way would be to inject (i.v.) a pre-determined amount of radiolabelled (^{125}I) mouse IgG or IgG + IgM either during or prior to stress. At different time points, after injection, the animals should be thoroughly perfused with PBS, their brain and spinal cord extracted and placed into tubes to be counted by a gamma counter. The percent of antibody that entered the parenchyma could be calculated by dividing the measured counts per minute (cpm) by the injected cpm and multiplied by 100. To determine where the antibody was most likely to cross, the above experiments should be repeated, tissue processed for sectioning and the sections subjected to autoradiography (injection of peroxidase labeled antibody rather than radiolabelled antibody and development with horseradish peroxidase may give similar results). Additionally, these experiments should be repeated using antagonists and agonist specific for the HPA axis and the SNS. Due to the negative feedback control of the HPA axis results obtained with corticosterone or dexamethasone administration should be take with caution as these steroids would expect to limit production of CRH from the hypothalamus, a hormone potentially implicated in BBB disruption. Collectively these studies should determine if stress increases neutralizing titers of TMEV specific antibody in the circulation, if circulating antibody levels are allowed

entry across the BBB in stressed and non-stressed mice, and provide a potential mechanism for altered entry of these antibodies.

6.2.2 The influence of stress-induced increase in antibody on the pathogenesis of TMEV infection

Because stress may alter the pathogenesis of Theiler's virus-induced demyelination by increasing intrathecal antibody production, studies should be devised to address this issue. These studies should follow the design of Sieve et al., (2004), and test for TMEV-specific antibody production in the brains and spinal cords of mice groups at days 30, 50, 100, 150 and 200 p.i. by ELISPOT. Additionally, ELISPOT assays should be designed to specifically determine subclass of antibody being produced behind the BBB, as those that are complement fixing would be seen as potentially detrimental. Moreover, the numbers of myelin-reactive antibody producing cells should also be determined in the same fashion. Finally, the pathogenic potential of CNS antibody producing cells should be determined by transfer experiments into naive mice. Finally, because B-cells are, in fact, professional antigen presenting cells, capacity of CNS-isolated B-cells to present both viral and myelin epitopes to T-cells should be determined using functional assays.

6.3 Determine the immunological relevance of RS on the polarization of different TMEV-specific T-cell subtypes

6.3.1 To characterize the effects of RS on Theiler's virus specific Th1, Th2, and Th17 responses from CD4+ T cells as well as CD11c+ dendritic cells *ex vivo* in SJL mice

During acute TMEV infection, virus specific $CD4^+$ T helper (Th) cells are protective as illustrated by the fact that the majority of $CD4^+$ deficient mice will die within days following injection. Restraint stress (RS) has been shown to limit immune responses to several viral infections including influenza and herpes simplex. Mechanistically, this effect is thought to stem from a Th1/Th2 shift in immunity. However, our data suggests that RS results in an overall immunosuppression that is reversible following the secession of RS. As such, we propose that over the course of 8 days of RS both dendritic cell function and $CD4^+$ Th responses (Th1, Th2 and Th17) will be decreased at days 4, and 8 p.i., but will recover and be comparable to those of non-stressed animals by day 16 and 24 p.i. The effects of RS on TMEV induced Th polarization in these experiments should be determined using purified CD11c+ dendritic cells isolated from the spleen, as well as $CD4^+$ T-cells isolated from the CNS and the spleen. The effects of stress on the polarization of $CD4^+$ T-cells should be demonstrated using, intracellular cytokine staining and flow cytometry, mRNA expression and western blotting of transcription factors, and by cytokine secretion. Moreover, the demonstration of virus induced activation of transcription factors responsible for T-cell polarization should be determined by ChIP assays.

6.3.2 To determine the roles of glucocorticoids and catecholamines on Theiler's virus induced APC activation and function *in vitro*

Antigen presenting cells (APCs) including macrophages and dendritic cells (DC) are not only required for the generation of adaptive immune responses, but are also thought to influence T helper cell polarization – thus making them essential components of the immune system. Dendritic cells, in particular, play an imperative role in uptake of antigen, and subsequent presentation, activation and polarization of naive $CD4^+$ T cells into effector $CD4^+$ T cells. Stress influences immunity through the activation of both the sympathetic nervous system (SNS) leading to the release of norepinephrine (NE), epinephrine (EPI) as wells as the hypothalamic-pituitary-adrenal axis resulting in the release of glucocorticoids. As APCs have been shown to express receptors for NE (β2-AR), EPI (β1-AR) as well as glucocorticoids (GR), studies should be designed to investigate the effects of these stress-induced molecules on the functional responses of these cells to Theiler's virus. Specifically, peritoneal macrophages or bone marrow derived DCs from SJL mice should be cultured with or without EPI, NE or DEX. These cells should then be sham infected with PBS or infected with TMEV at various multiplicities of inefection. At 5, 24 and 48 hours post infection (p.i.), APC function will be assessed by mRNA expression, cytokine secretion (IL-23(p19), IL-12(p70), IL-6, and IL-10), and presence of activation markers (MHC class II, CD80, CD8α). Additionally, the function of these APCs should also be assessed by their ability to stimulate naive CD4+ T cells into effector cells (Th1, Th2 or Th17).

6.4 Determine the relevance of T-bet in the demyelination disease of TMEV

While many studies have addressed the effects of IFN-γ on Theiler's virus-induced demyelination (TMEV-IDD) as well as its activator STAT4, no studies have elucidated the role of T-bet in TMEV-IDD. While T-bet is responsible for Th1 polarization and IFN-γ secretion it as so controls the regulation of IL-23R expression, indicating an involvement in propagating Th17 cells. In fact, while IFN-$\gamma^{-/-}$ mice developed more severe EAE, T-$bet^{-/-}$ mice on the same background are resistant. Given the requirement for Th17 cells in the facilitation of EAE it is tempting to speculate that these cells may be required for the induction of TVID. There is evidence that supports such a hypothesis: IFN-γ-/- mice on a normally TVID-resistant background are rendered susceptible to demyelination (Rodriguez et al., 1995), but TMEV infection cannot be confined to inducing either Th1 or Th2 immune responses (Monteyne et al., 1999). Also, macrophages from the highly susceptible SJL mouse strain have been shown to secrete high amounts of IL-12(p40), but low amounts of IL-12(p35) whereas the macrophages from resistant mice secrete low amounts of IL-12(p40) (Petro, 2005a). Moreover, TMEV infection of the macrophage cell line, RAW264.7, induces expression of IL-23 (Petro, 2005b). Additionally, we have found that TMEV infection increases serum concentrations of IL-12(p40), but has no effect on IL-12(p70) concentrations, and mice possess Th17 cells in the CNS at day 8 p.i. in SJL mice as well as high quantities of IL-17A producting cells in the spleens during chronic disease (not shown).

Furthermore, both IL-6 mRNA expression (Mi et al., 2006b) as well as protein concentration (Johnson et al., 2006; Steelman unpublished observation) is increased following infection with the BeAn strain of TMEV, and MIP-2 (CXCL2), a protein induced by IL-17, is also found to be increased during the demyelinating phase, and maybe involved in susceptibility (Rubio et al., 2006). Finally, the abolition of IL-12(p40) via monoclonal antibody administration during acute infection delays demyelination, but STAT4$^{-/-}$ mice on a normally TVID-resistant background are rendered susceptible to demyelination (Inoune et al., 1998; Rodriguez et al., 2006). Therefore, it will be interesting to determine the effects of T-bet on the pathogenesis of TMEV infection. Studies utilizing T-bet$^{-/-}$ mice on a resistant C57BL/6 background should be used to examine the course of TMEV infection. The effect of virus specific immune responses in T-bet$^{-/-}$ mice on other T-cell subsets should also be explored. Subsequent studies should be used to determine the mechanism of observed effects (i.e. death and/or exacerbated disease).

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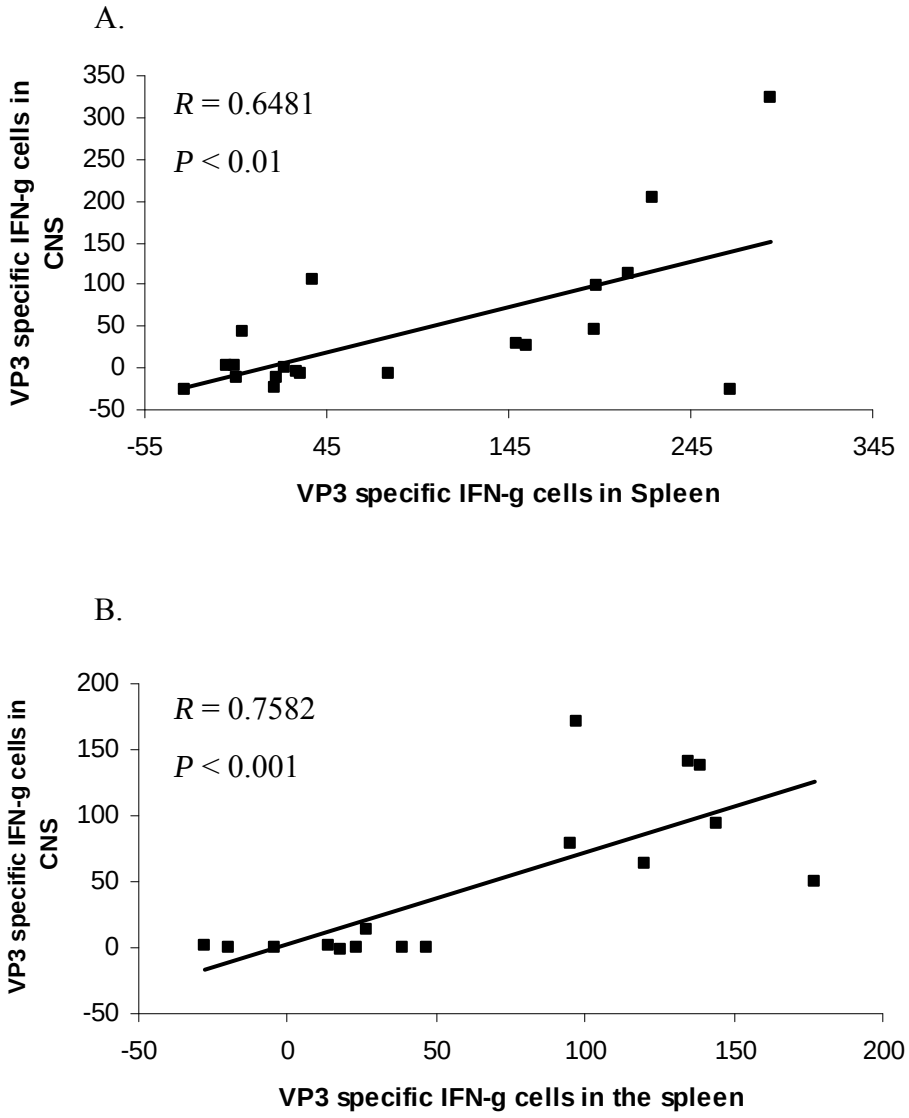
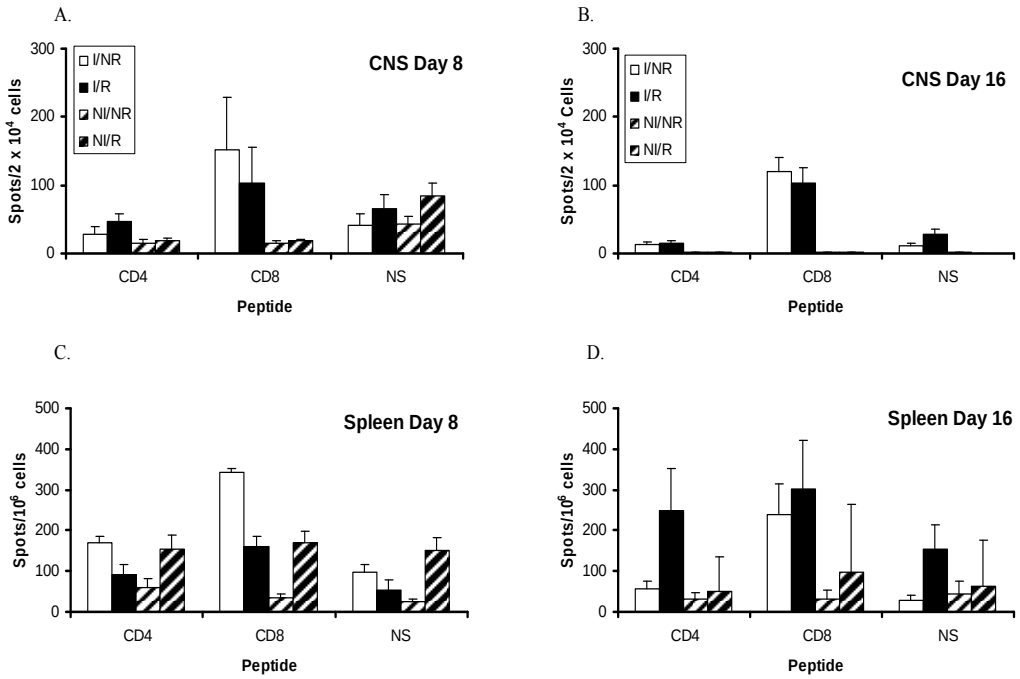
APPENDIX A: CORRELATIONS



Appendix A. The number of IFN-γ producting virus specific $CD8^{+}$ T-cells were correlated between the CNS and spleens at both days 8 p.i. (A), and 16 p.i. (B).

APPENDIX B: ELISPOT RAW DATA



Appendix B. Elispot Raw Data. A-D. Number of IFN-γ producing cells counted from the CNS (top) or spleens (bottom) of infected/non-restrained (I/NR), infected/restrained (I/R), non-infected/non-restrained (NI/NR), and non-infected/restrained (NI/R) mice at days 8 p.i. (A,C) or 16 p.i. (B,D). To ensure $CD4^+$ and $CD8^+$ T-cell specific IFN-γ production, the background spots, determined by incubation with a non-specific peptide sequence (NS), were subtracted from the numbers generated by incubation with both $CD4^+$ and $CD8^+$ immunodominant peptides from each individual mouse. Data are means ± SEM.

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